STUDIES ON INFECTION BY AND RESISTANCE TO THE PREISZ-NOCARD BACILLUS[1]

2. SUSCEPTIBILITY OF THE GUINEA-PIG, AND THE DISTRIBUTION OF LESIONS AFTER CUTANEOUS AND SUBCUTANEOUS INOCULATION AND INGESTION.

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In order to determine what variation, if any, has resulted from attempts to immunize guinea-pigs against infection by the Preisz-Nocard bacillus one must first have some knowledge of the disease in unimmunized animals. Although this subject has not been pursued systematically, sufficient data have been accumulated to enable us to draw some conclusions as to the result of experimental infection by several methods in the guinea-pig.

While results of experimental infection in the guinea-pig cannot be applied uncritically to the disease, caseous lymph-adenitis, in sheep, the basic principles involved in the biological reactions to the infection may reasonably be assumed to be similar in these two mammalian species. Therefore another aspect may legitimately arise, although only incidentally, from these studies.

The pathogenicity of the Preisz-Nocard bacillus in the guinea-pig has been studied by many workers, particularly those in France. It is well known that relatively large doses (1·0 cc. to 0·1 cc.) of young cultures of the micro-organism when injected into guinea-pigs will produce death within a few hours to a few days, and that less toxic cultures will give rise to suppurative lesions. Most attention has been given to the study of toxic death resulting from the injection of cultures or the soluble toxin and to the production of lesions in the genital organs of male guinea-pigs.

The pathogenicity of the cultures used by various workers has varied very much, and there is little or no evidence in the literature that any special precautions have been taken to preserve the pathogenicity of stock cultures. Panisset

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(3) in 1910 made a special study of the infection in the guinea-pig. He employed intraperitoneal, subcutaneous, and intravenous inoculations of bacillary emulsions. Taking the centigramme as his unit of dosage, he found that doses of 10^{-5} were necessary for regular infection of the genitals in male guinea-pigs when the inoculation was made intraperitoneally, although lesions were produced less regularly by doses of 10^{-7}. In all cases death followed inoculation, but varied in its occurrence according to the dose, occurring in $3\frac{1}{2}$ days following a dose of 10^{-1} and in 12 days following a dose of 10^{-6}. He described the evolution of the infection of the *tunica vaginalis* in these animals. He found the female guinea-pigs more resistant to intraperitoneal inoculation than the male. With doses 10^{-2} and 10^{-3} all the female animals survived and all the male animals died. He found also that young animals were more resistant than adults. Subcutaneous inoculation of doses from 10^{-5} to 10^{-9} produced local abscesses and involvement of the lymphatic glands. The abscesses usually broke down and discharged before finally healing after five or six weeks. Intravenous inoculation, by direct injection into the heart of doses from 10^{-1} to 10^{-3}, produced death in from 5 to 19 days. An eruption of pustules in the skin occurred the day following inoculation. This dried up, but after several days a new eruption appeared.

Hall and Stone (2) in 1916 studied the pathogenicity of cultures isolated from equine, bovine, and ovine abscesses. They made special mention of the production of orchitis in the male guinea-pig, and found the most prominent visceral lesions to occur in the omental lymph nodes and the spleen.

The present communication deals mainly with the anatomical distribution of lesions following the inoculation of small doses of virulent cultures and a comparison of the distribution of the lesions following several modes of infection. We are not concerned at this time with the lethal effect of cultures or soluble toxin. Most of the animals were used in experiments to determine the minimal infective dose of cultures that had been kept in alkaline egg medium and subcultured on hormone agar, as described in a previous communication (1), the inoculum being a bacillary suspension in bile-saline solution.

Subcutaneous Inoculation.

A study has been made of the distribution of lesions in 308 guinea-pigs inoculated subcutaneously in the thigh region with varying doses of a bacillary suspension of the Preisz-Nocard bacillus. Forty-five (14·6% of the whole) of these animals were inoculated with a bacillary suspension prepared by taking a 24 hours old subculture on a blood agar slant ($\frac{3}{4}$ inch tube) and suspending the growth in 5 cc. of 0·85% sodium chloride solution. The doses varied from 1·0 cc. to 0·00312 cc. Most of the other animals were inoculated with a bacillary suspension in 0·5% or 0·1% sodium chloride solution containing 0·12% of bile

salt. The doses varied from ten or less to hundreds of thousands of bacilli and rarely more.

Apart from the production of a local lesion and the involvement of the popliteal gland, the following organs and tissues contained lesions in the proportions indicated.

	Times.	Percentage.
Inguinal lymph gland	109	35·3
Precural lymph gland	73	23·7
Iliac lymph gland	101	32·7
Liver	129	41·8
Spleen	115	37·3
Bones and joints	11	3·5
Pericardium	4	1·2
Peritoneum	8	2·6
Testicle	2	0·6
Kidney	2	0·6
Lungs	2	0·6
Prescapular lymph gland	3	0·9
Axillary lymph gland	5	1·6
Mediastinal lymph gland	5	1·6
Paraortic lymph gland	6	1·9
Retropancreatic lymph gland	2	0·6
Cervical lymph gland	1	0·3

The extent of the generalization of infection naturally depends largely on the time elapsing after the inoculation. Some of the animals died, but the majority were destroyed in from 21 to 30 days after inoculation. The examinations were made between the extremes of 11 and 107 days after inoculation.

Following subcutaneous inoculation with relatively small doses of washed bacilli suspended in bile-saline solution a swelling is noticed at the site of inoculation in four or five days' time. If the inoculation is purely subcutaneous the lesion usually breaks down and discharges to the outside in about fourteen days or longer.

The size of the secondary lesions has varied greatly, the largest abscess being one developing in the margin of the liver and attaining the size of a billiard ball. Local abscesses always develop at the site of inoculation, and frequently break down and discharge to the outside. Usually the lymph glands draining this area become involved, but not invariably. Out of the 308 animals inoculated 44 (14·3%) showed no involvement of the precrural, inguinal, or iliac lymph glands, but showed lesions in the distant organs, usually including liver or spleen. It is evident, therefore, that a small proportion of the animals may show no involvement of the lymph glands draining the areas of the primary lesion. Should this lesion discharge to the outside with subsequent resolution, as occurs occasionally (observed definitely in 3·5% of cases and probably in 12%), there may be no evidence of the method of entry of the micro-organism when the animal is examined *post mortem*.

There appears to be little general effect from the disease and little loss of

weight, and if the animal is allowed to live generalization always develops. The liver and spleen are most frequently involved, but bones and joints only occasionally. The lungs have only twice been found to be involved following subcutaneous injection.

Included in this group of animals inoculated subcutaneously are 12 guinea-pigs which were inoculated with a 1 in 10 saline suspension of purulent material from a natural lesion in the gland of a sheep. The doses ranged from 1 cc. to 0·1 cc. Four of the animals were killed and examined after 22 days and eight after 16 days. Local lesions were produced in all, but in none were the inguinal or precrural glands involved. In only two of them were the iliac glands involved, representing only half the average number for the group as a whole. The spleen was involved in eleven of these animals and the liver in six.

Also included in this group are 12 animals (3·8% of the whole) which were inoculated with varying doses of toxin (centrifuged broth cultures or plaster of Paris filtrates) which were found to be not sterile. The course of the disease does not appear to have been different from the average of the group as a whole. The number of bacilli inoculated must have been very small, and it is almost certain they would have been present in small clumps of three or four or more micro-organisms. The smallest dose given was 0·0156 cc. of a plaster of Paris filtrate, and the animal died in 60 days, showing lesions in the liver, iliac gland, right testicle, and inguinal gland.

One other case is worthy of mention, although it is not included in the group. The circumstances associated with the infection of this animal are exceptional, and the disease became more generalized than in any other animal coming under our observations. It received subcutaneously 1 cc. of a plaster of Paris filtrate which had had 0·5% of formalin added and had been kept at 37°C. for 7 days in a sealed ampoule. Either the animal was resistant to the action of toxin or the toxin had been destroyed. Death resulted in 21 days. At the site of inoculation there was a large abscess; the inguinal glands were involved on both sides, and the precrural and axillary glands were involved on the right side. There were abscesses in the intercostal muscles on both sides, and the submaxillary and prescapular glands were involved. Lesions were also present in the paraortic glands, liver, spleen, and left lung. In all, there were thirty-two foci scattered throughout the body.

It is apparent from this study that however small the dose of bacilli given subcutaneously, a local lesion always develops and persists except in a small proportion of cases, when, following complete evacuation, resolution occurs. There is also rapid involvement of the regional lymph glands, liver, and spleen.

Infection of Wounds.

A small group of guinea-pigs were infected by incising the skin of the insides of the thighs with a sharp pair of scissors, giving a wide V-shaped wound

extending into the subcutaneous tissues and leaving them more or less exposed, due to the retraction of the skin. Twenty-two animals in all were treated in this way. At first 8 were so treated, and the wounds smeared with purulent material from a natural lesion from a sheep. The wounds in each case suppurated, but later healed without leaving any lesion. The animals were killed 35 days later, when lesions were found in the following situations:

	Times.	Percentage.
Precrural lymph glands	6	75
Inguinal lymph glands	1	12·5
Cervical lymph glands	6	75
Popliteal lymph glands	0	0
Iliac lymph glands	0	0
Liver	5	62·5
Spleen	5	62·5
Testicle	1	12·5

Another lot of 14 animals were treated in a similar manner some time later, but the inoculation was much smaller. Half of them received one drop of a 1 in 10 dilution of purulent material from a sheep, and the other half one drop of a 1 in 100 dilution, on the wounded surface. The mouths of the animals were tied up for some time after the inoculation, as the high percentage of involvement of the cervical glands in the previous group suggested that the animals had licked the lesions and taken infected material into the mouth. The animals were killed 35 days later, when lesions were found in the following situations:

	Times.	Percentage.
Precrural lymph glands	10	71·4
Inguinal lymph glands	0	0
Cervical lymph glands	2	14·2
Popliteal lymph glands	6	42·8
Iliac lymph glands	1	7·1
Liver	8	57·1
Spleen	6	42·8
Parotid	1	7·1

Although the cervical lymph glands infected were much reduced in numbers in the second group, their involvement was not entirely prevented, as the securing of the mouth was not completely successful. One animal of the second group failed to show any lesion after the wound had healed.

It is evident that the guinea-pig is highly susceptible to contact with a small quantity of contagium from the sheep, and there appears to be less tendency to involvement of the inguinal and iliac lymph glands than after subcutaneous inoculation of bacilli from artificial cultures.

Intracutaneous Inoculation.

Fourteen guinea-pigs became infected by the intracutaneous inoculation of unfiltered toxin which had been centrifuged to remove the bacilli. These results were incidental to some attempts made at the titration of toxin by the intradermal method. The dose in each case was 0·1 cc. of toxin or diluted toxin, and the number of micro-organisms present must have been very small. Inoculations

were made in the skin of the abdominal wall, each animal receiving several injections, usually eight.

When a local lesion was produced the skin at the site of inoculation became necrotic, and a small, punched-out ulcer from 2–3 mm. in diameter developed.

Of these 14 animals, 8 presented purely local lesions at the time of examination, but 6 had developed lesions in distant tissues or organs. The liver was found involved three times, the precrural, mesenteric, axillary, and iliac glands once each, and the omentum once.

These results illustrate the high infectivity of unwashed bacilli in the presence of toxin. The number of bacilli present would be very small, probably in the form of a small clump that had escaped being thrown down in the centrifuge. Another point of interest is that among these animals the only example of involvement of a mesenteric lymph gland is to be found.

Intraperitoneal Inoculation.

This method of inoculation has been used by others to illustrate the frequency with which the *tunica vaginalis* becomes the site of lesions. Fourteen animals were inoculated with a dose of 0·1 cc. of bacillary suspension prepared from blood agar slants as mentioned above. Three of the animals died within 3 days and the remaining 11 were killed approximately 30 days later. In these, lesions were present in the following tissues: precrural lymph gland once, iliac lymph gland once, tunics of the testicle three times, spleen once, omentum six times, and liver four times, and peritoneal abscesses were found six times.

The tunics of the testicle were infected in a lower proportion of cases than has been found by most other workers, but we did not make an examination of these tissues except when gross lesions were present. In one animal spontaneous discharge of the lesion in the *tunica vaginalis* occurred, and when examination was made *post mortem* some days later complete resolution was found to have taken place.

Infection by the Mouth.

In the earlier stages of these studies a number of guinea-pigs were drenched with saline suspensions of a 24 hours old subculture of the bacillus on blood agar. The suspension was the same strength as that made from blood agar slants mentioned above. In all 99 animals were drenched, the dose varying from 1·5 cc. to 0·062 cc. Of these, 59 animals became infected. Low infectivity was due in one instance to the use of an old stock strain which had lost some of its pathogenicity through being kept on alkaline-egg medium too long without subculture. With the use of a new strain the infections rose to 71%. Not all guinea-pigs show the same susceptibility to infection by a single administration of bacilli by this route. In one experiment with four animals in each group, 100% in one group were infected after being drenched with 0·062 cc. of the suspension, while only 50% were infected after 0·5 cc.

The doses are relatively very large, but it is believed that much smaller doses would lead to infection, particularly if repeated on several occasions.

The 59 animals that became infected showed involvement of the following tissues and organs:

	Times.	Percentage.
Cervical and submaxillary lymph glands	30	50·8
Prescapular lymph glands	2	3·3
Axillary lymph glands	4	6·7
Retropancreatic lymph glands	2	3·3
Precrural lymph glands	6	10·1
Mediastinal lymph glands	5	8·4
Popliteal lymph glands	1	1·6
Lungs	12	20·3
Liver	18	30·5
Pericardium	2	3·3
Diaphragm	1	1·6
Spleen	9	15·2
Bones and joints	7	11·8
Muscle	1	1·6

Approximately half of the animals showed involvement of the cervical or submaxillary lymph glands. This involvement did not appear to occur less frequently in those receiving the smaller doses.

The distribution of the lesions in some of the animals is of particular interest. In three animals involvement was confined to the superficial lymph glands. Of these three, one showed involvement of the left precrural gland only, another showed involvement of the prescapular and precrural glands of the right side only, and the third of the right prescapular gland only. Two animals showed involvement of the mediastinal glands only, and one showed involvement of the right elbow and carpus, as well as the left popliteal gland.

Natural Infection.

There remain two animals which deserve mention. These are classed under natural infection, although only one of them can strictly be so placed.

In the one case an experiment was conducted by making an emulsion of purulent material from a natural lesion of a sheep, and an area of ground covered with grass and weeds was sprinkled with this emulsion. Two guinea-pigs were allowed to graze over this area for 4 days, when they were removed and kept in a cage. They were killed a month later. One animal showed an abscess in the axillary gland as the sole lesion, and the other showed no evidence of infection.

In the other case an infected female guinea-pig was kept in a cage for some time, during which it gave birth to a single offspring. When this animal was three months old it was found to have a discharging prescapular abscess, involvement of a precrural gland, a large abscess in the liver 1 inch in diameter, and another large abscess $1\frac{1}{2}$ inches in diameter in the omentum. The Preisz-Nocard bacillus was recovered in cultures from these lesions.

Both of the animals in this group almost certainly became infected by the ingestion of contaminated material.

COMMENTARY.

These results are outlined mainly as a record of the lesions that may result from infection of the guinea-pig by the Preisz-Nocard bacillus. This animal probably differs from other animals in the susceptibility of certain tissues, notably those of the liver and spleen. In this respect, the same susceptibility is found in experimental tuberculosis. The lymph glandular tissues are particularly susceptible, but the lungs are rarely involved, except in those animals drenched with bacillary suspensions made from young cultures.

When the animals are inoculated subcutaneously or infected through wounds in the skin, the lymph glands draining these areas are involved in the large majority of cases.

When the animals are infected by ingestion of cultures, only half of them show involvement of the cervical or submaxillary lymph glands, in these cases infection probably taking place through the mucous membrane of the mouth or pharynx. When the amount of infected material is small, and particularly when it is intimately associated with ingested food, this involvement of the cervical and submaxillary lymph glands may be absent.

It is clear that involvement of the superficial lymph glands, particularly the precrural and prescapular, may occur without infection being introduced into the tissues drained by these glands.

Infection appears to be almost solely lymph borne, and the bacilli are probably always carried by phagocytes. At no time have we been able to obtain a growth of the bacillus from heart blood collected *post mortem*.

The experiments were not designed for the purpose of throwing light on the possible mode of infection of sheep under natural conditions. It is possible, however, that the results may indicate the danger of concluding that the sole or main cause of involvement of the superficial lymph glands is infection of the tissues drained by these glands. It is interesting to note, also, that in no case was involvement of the mesenteric lymph glands found when infection was produced by ingestion. It has often been claimed that natural infection of the sheep by ingestion is not at all likely, because lesions in the mesenteric lymph glands are rare in this animal.

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NEW TREMATODES FROM THE SUBANTARCTIC AND ANTARCTIC

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PSEUDOBENEDENIA NOTOTHENIAE n.g., n.sp.

In 1907 Professor W. B. Benham, F.R.S., collected several ectoparasitic trematodes gliding over the body of certain fishes from Antipodes Island, one of the subantarctic islands of New Zealand. He kindly forwarded his material to the writer, the specimens being labelled as having been obtained from *Notothenia angustata* and *N. colbecki*, the identification of the hosts being made by the late E. R. Waite, who subsequently considered *N. angustata* to be a synonym of *N. macrocephala*. These parasites were identified by Dr. Benham as *Tristoma* sp., and recorded by Waite (3), who mentioned subsequently (4) that they were similar to those occurring on *Notothenia macrocephala* at Macquarie Island.

An account of the species, based on the material from Antipodes Island, together with that collected from the last-named fish by the Australian Antarctic Expedition of 1911-1914 at Macquarie Island, has been awaiting publication for some years. It is proposed to give a short description of it now, leaving the detailed account to appear later.

The worms are flat, semitransparent, and elliptical, measuring from 5·5 to 6 mm. long (excluding the disc) by 4 to 4·7 mm. in breadth. If the disc be included, the length is 6·5 to 7 mm. The largest compressed specimen was 7 by 4 mm. The rounded disc, which is nearly circular, with a diameter of from 2 to 2·4 mm., is connected with a deeply indented part of the body by a short, narrow pedicel.

On each side of the parasite, near the corresponding anterior sucker, is a well-defined notch marking the posterior limit of the anterior glandular area. The latter are homologous with the so-called suckers of *Epibdella (s.str.)*, and appear as rounded lobes which merge anteriorly. There is also a well-developed anterior sucker on each side ventrally, about 0·8 mm. in diameter, between the mouth and the glandular areas, and connected with the body by a short, narrow stalk. The mouth is large and crescentic and in line with the rear margin of the

suckers. Just behind the left sucker there opens the common genital aperture, while the vagina terminates on the same side some distance behind the latter and somewhat nearer the midline. The excretory pores open dorsally on either side, a little behind the level of the genital opening.

The disc possesses two small notches posteriorly, one near each member of the third pair of hooks. It is highly muscular, except for a thin marginal area. The first pair of hooks are much shorter and thicker than the long, narrow, curved second pair, and have their axes inclined so that their free anterior points face slightly laterally. The second pair end each in a strongly curved projecting hook. The axes of the second and third hook of each side are directed towards the indentation at the end of the disc on the corresponding side. The third pair are very small, with a relatively flat broadened portion and a terminal hook which projects just behind that of the second. The three hooks measure 0·9–0·23 mm., 0·38–0·53 mm., and 0·13–0·17 mm. respectively. There are also numerous small, rounded, highly refracting bodies disposed in a number of more or less symmetrical groups arranged in the vicinity of the paired hooks.

The disc bears six slight ridges, apparently homologous with the antero-lateral, posterior, and postero-lateral septa in *Capsala*, the posterior lying below the second and third hooks, the postero-lateral curving outwards and backwards from the first pair, while the antero-lateral pass forwards and outwards from the region of the pedicel. These ridges are not muscular, and do not lead to the development of definite loculi.

The forwardly directed mouth opens into a crescentic buccal cavity, into which projects part of the very prominent pharynx. The latter contains numerous large gland cells whose secretion stains readily. The organ is much like that described by Heath for *Epibdella squamula*. The very short oesophagus receives the long narrow ducts of abundant salivary cells, which occupy a triangular area on each side postero-laterally from the pharynx. The intestine is very extensive, its branches extending above most of the other organs. The two main crura bifurcate just behind the pharynx into a short anterior and a long posterior branch, each giving off inwardly, as well as laterally, diverticula which divide more or less dichotomously several times, and terminate near the edge of the parasite. The main branches closely invest the outer and posterior edges of the testes. Coalescence of the two crura was not observed.

The rather large brain lies just in front of the pharynx, and bears two pairs

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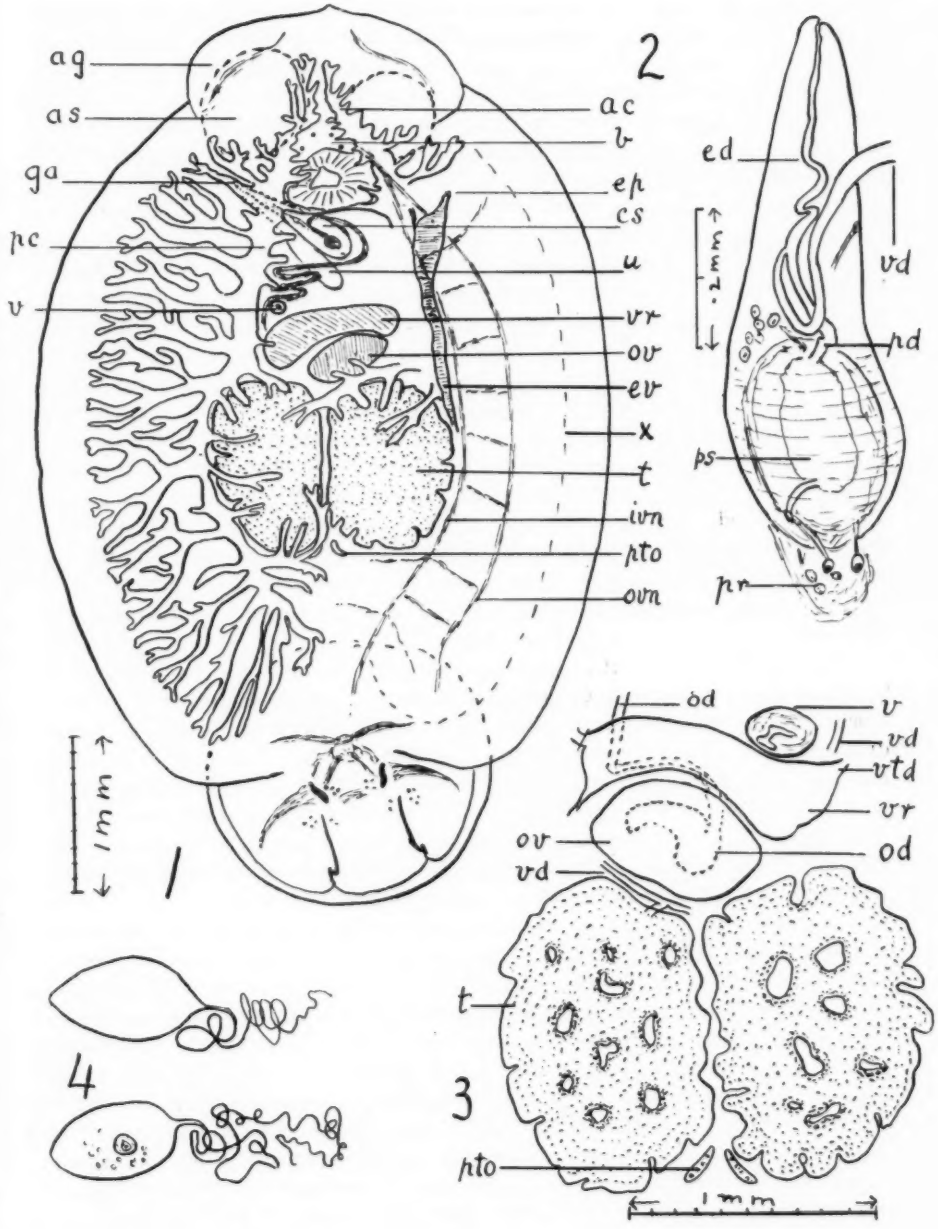
Figures 1-4. *Pseudobenedenia nototheniae.* Figure 1: entire worm, dorsal view, vitellaria omitted. Figure 2: cirrus sac. Figure 3: genital organs, ventral view. Figure 4: eggs.

References to lettering: ac, anterior crus of intestine; ag, anterior glandular area; as, anterior sucker; b, brain with eyes; cs, cirrus sac; ed, ejaculatory duct; ep, excretory pore; ev, excretory vesicle; ga, genital aperature; ivn, inner ventral nerve; od, oviduct; ov, ovary; ovn, outer ventral nerve; pc, posterior crus; pd, prostate duct; pr, prostate; ps, prostate reservoir; pto, post-testicular organ; t, testes; u, uterus; v, vagina; vd, vas deferens; vr, vitelline reservoir; vtd, vitelline duct; x, outer limit of intestine and vitellaria.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of eyes dorsally. There is well-marked bilateral disposition of its nerve cells. In front of the brain is a strong anterior commissure. The longitudinal nerves are the dorsal, inner ventral, and outer ventral on each side, with many commissures between the ventrals.

The main excretory canals lie close above the corresponding inner ventral nerve trunk. There is a large elongate vesicle on each side, just laterally from the zone occupied by the ovary and sex ducts, its external aperture lying nearly midway between the margin and the midline of the parasite at the level of the pharynx.

There are two large testes, side by side, each measuring 1·6 to 1·2 mm. long by 1·0 to 0·8 mm. broad, the sizes varying with the degree of compression. Each is more or less deeply indented marginally and perforated by numerous strands of medullary tissue. The vas deferens arises from anterior portions of the glands, passes to the left behind the ovary, curves in front of the large vitelline reservoir, winds about in the region in front of the latter, and extends forwards above the uterus and cirrus sac. It is now a narrow tube, which travels backwardly to enter the cirrus sac laterally, and eventually joins the prostate duct to form the ejaculatory duct, which is relatively thick-walled. The penis is not chitinized. The pyriform sac, which is compact and resembles that of *Epibdella ovata*, contains a spherical prostate reservoir. Prostate gland cells are abundant in the region occupied by the vas. Close behind the testes are two deeply staining post-testicular glands, like those reported for certain species of *Epibdella*.

The ovary is a compact organ, measuring about 0·5 to 0·65 mm. across and 0·35 mm. long, with a wide oviduct issuing more or less centrally from it after a bent course within the gland. The oviduct is soon joined by the common yolk duct, and then passes forwards above the right side of the vitelline reservoir and above the vas deferens to become the ootyp, which is a conspicuous organ with a chitinized wall and wide lumen. It receives numerous long ducts from shell glands, and then becomes narrowed to enter the uterus, which possesses an external sheath of loose, feebly-staining tissue. Within this the duct becomes bent and markedly widened, and then enters the genital atrium.

The yolk follicles are very numerous, and lie dorsally amongst the branches of the intestine, extending from the most anterior to the most posterior caeca. There is a large anterior and posterior collecting duct on each side near the corresponding intestinal crus, the four entering separately into the conspicuous transversely placed reservoir which lies just in front of the ovary. Near its midline arises the common yolk duct. The vagina is a very short, wide tube, with very thick muscular walls and a cavity lined by abundant hairs. It opens ventrally on the left of the midline just in front of the yolk reservoir and at a considerable distance behind the common genital aperture. Only one fully formed egg is present at a time in the uterus. It measures 0·2 mm. by 0·1 mm., and terminates in a very long filament, which may reach 1·2 mm. in length.

In a recent paper (Johnston, 1) it was shown that the name *Epibdella* must be submerged as a synonym of *Entobdella* Blainville, and that, as Odhner had previously indicated, *Benedenia* should be restored to include those species, formerly listed under *Epibdella*, but which are provided with two anterior suckers instead of anterior glandular organs. These two genera were subdivided into subgenera, according to the position of the vagina in relation to the other sex ducts.

The species described above differs from other *Epibdella*-like trematodes in possessing both anterior suckers and anterior glandular areas, as well as in certain other features. The differences being regarded as of generic value, the name *Pseudobenedenia* is suggested for the proposed new genus.

Generic diagnosis: Capsalidae; possessing a pair of anterior suckers in addition to well-marked anterior adhesive organs; an extremely short vagina, remote from the other sex ducts; prostate reservoir lying within the penis sac; testes perforated; posterior disc with indications of three pairs of non-muscular septa; remaining characters as in *Entobdella* and *Benedenia*. Type, *P. nototheniae* n. sp. from species of *Notothenia*.

Epibdella, using the term in its former wide sense, i.e., to include the species variously placed under *Phyllonella*, *Epibdella*, *Phylline*, *Entobdella*, and *Benedenia*, is usually regarded as belonging to the subfamily Tristomatinae (i.e., Capsalinae), but Monticelli in 1902 placed it under a new subfamily, Ancyrocotylinae, along with *Ancyrocotyle*. The latter genus, however, is characterized by the presence of a different type of anterior glandular organs and anterior suckers; by the possession of a single testis which is pre-ovarian; by the absence of lateral caeca along each intestinal crus; and by the absence of a pair of hooks corresponding to the first pair of Epibdellids.

In *Tristoma* (i.e., *Capsala*) there are numerous testes; the posterior sucker is sessile, and possesses seven well-marked radii, subdividing it into seven lateral loculi, in addition to a central loculus; while its hooks are different from the Epibdellid type. Though the remainder of the organography is arranged on the same general plan, it seems advisable to restrict the Capsalinae to include only genera, such as *Capsala*, possessing the characters just referred to, and to separate from it the group of genera and subgenera of the Epibdellid type, for which a new subfamily, Benedeniinae, is now proposed, and the following diagnosis suggested:

Capsalidae with large stalked posterior sucker, provided with, typically, three pairs of hooks, the second pair largest, the third smallest (or perhaps absent), but without muscular septa subdividing it into loculi; either with anterior glandular organs *(Entobdella)*, or with anterior suckers *(Benedenia)*, or with both *(Pseudobenedenia)*; with two testes lying side by side.

The subfamily, Ancyrocotylinae, should be restricted to contain forms like *Ancyrocotyle*, devoid of lateral intestinal caeca; but having a single preovarian

testis; a pair of very long hooks, and a pair of very small hooks on the disc, which is devoid of septa; and peculiar anterior adhesive apparatus. This subfamily resembles Encotyllabinae in the general characters of its disc and anterior adhesive organs, as well as the simple bifurcated intestine; but the arrangement of the reproductive system in the latter subfamily is essentially similar to that of Benedeniinae.

Nitzschia resembles the Epibdellid type of trematode in possessing anterior adhesive organs which simulate suckers; a large posterior disc with a short stalk, without radii or loculi, but with three pairs of small hooks obviously representing those of *Entobdella* or *Benedenia;* and branching intestinal caeca arising from the crura. It differs, however, in having numerous testes, and in the median position of its genital openings, as well as in the absence of a vagina. These special characters justify the separation of these sturgeon parasites from the Capsalinae, where they have previously resided, and their inclusion in a distinct new subfamily, Nitzschiinae, containing the single genus, *Nitzschia.*

The genera and subgenera of the subfamily Benedeniinae may be distinguished as follows:

A. True anterior suckers absent, anterior adhesive organs present, *Entobdella.*
Vaginal opening closely adjacent to the other genital apertures, subgenus *Parepibdella.*
Vaginal opening not beside the other genital apertures, subgenus *Entobdella.*

B. True anterior suckers present, anterior adhesive organs present, *Pseudobenedenia.*
True anterior suckers present, anterior adhesive organs absent, *Benedenia.*
Vaginal aperture beside genital openings. Subgenus *Benedenia.*
Vaginal aperture not beside genital openings. C.

C. Vagina travelling more or less directly forwards from yolk reservoir to vaginal aperture. Subgenus *Parabenedenia.*
Vagina very long, and travelling posteriorly before passing forwards to its external aperture. Subgenus *Benedeniella.*

A parasite somewhat resembling *P. nototheniae*, at least in externals, was described by Braun as *Lophocotyle cyclophora.* It was collected from the body of a fish, probably a species of *Notothenia*, at Navarin, Tierra del Fuego. Its male system, as well as the structure of the posterior disc and of the anterior region, are quite different, though the vitelline, excretory and digestive systems show a good deal of similarity.

Ogmogaster antarctica n.sp.

A number of trematodes were collected from the intestine of Weddell seals, *Leptonychotes weddelli*, at Commonwealth Bay, King George V Land, by the late Dr. A. L. Maclean during the sojourn of the Australian Antarctic Expedition. The species had been collected also by the Terra Nova Expedition (2), and was identified as *Ogmogaster plicata*, originally described by Creplin, and later by Jagerskiold, from northern whales, but it differs in a number of points, and is herein regarded as a new species. A more detailed account is awaiting publication in the Expedition's Reports.

The specimens are more or less cupped, with the margins inturned ventrally. They are thick, nearly rounded, measuring about 5–6 mm. long by 3·5–4 mm. broad, but one which was nearly flat was 6 mm. by 4 mm. The largest, whose edges were not turned in, measured 6·7 mm. by 5·5 mm., so that the species is smaller and wider and more rounded than Creplin's. The maximum width is at about the mid-length. The free margin is regularly lobed, 18 to 20 on each side, the folds being longest laterally, and merging into a smooth, transverse fold lying between the oral sucker and the sex opening. The ventral surface has thirteen deep furrows separated by prominent rugae, the outer ridges being shorter. These rugae do not reach the level of the genital pore, but they extend posteriorly almost to the end of the worm. The median ridge passes forwards between the long cirrus sac.

The oral sucker is about 0·5 mm. in diameter, and just behind the mouth is the common genital aperture, through which the long, spiny penis may be seen protruded. The digestive system is essentially like that of *O. plicata*, except that the curves of the crura are more nearly equal.

The testes are symmetrical, deeply incised organs, measuring 0·7–0·9 mm. by 0·6–0·7 mm., and lying outwardly and slightly anteriorly from the ovary. The cirrus sac is 1·8 mm. long by 0·34 mm. wide, and lies in the midline. It is thus about one-third the body length. The ovary is lobed, median, posterior, and more or less surrounded by the terminal portion of the crura. It measures 0·6 mm. across by 0·4 mm. in length. The uterus is similar to that of *O. plicata*, and ends in a short metraterm, about as wide as the cirrus sac, below the anterior end of which it lies. The female aperture is wide, muscular, and spiny. The yolk glands are more restricted than in the other species, and occupy a relatively narrow transverse zone in front of the shell gland and testes, extending forwards for about one-third of the distance between the shell gland and the posterior end of the cirrus sac. There are from 10 to 18 follicles on each side. The very abundant eggs measure about 0·02 mm. by 0·012 mm., excluding the tapering filament, but if it be included, the total length is about 0·18 mm.

O. antarctica from antarctic seals differs from *O. plicata* from northern

Balaenopterid whales in its smaller size, different body proportions, presence of only thirteen rugae, more restricted vitelline zone, and relatively smaller cirrus sac.

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ARE THE SKELETAL MUSCLES OF THE EXTREMITIES DIRECTLY INNERVATED BY SYMPATHETIC NERVES?

by

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The belief in a double innervation, somatic and sympathetic, of the striated musculature of the mammalian limb is due mainly to the work of Agduhr (1), which is an extension of the researches of Boeke (2) on the double innervation of the extrinsic muscles of the eye. Agduhr's conclusions have been supported by Kuntz (7), but Hinsey (6) was unable to find terminations of sympathetic nerves upon the striated muscle fibres of the limb, and Wilkinson (12), who had the privilege of studying Professor Agduhr's preparations, was unable to concur with the interpretation put upon them.

A clear demonstration of sympathetic nerves ending on muscle fibres of the mammalian limb would be specially desirable, since experiments designed to detect the effect of sympathetic nerves upon muscle have been performed mainly on this material; and also because the surgical operation of ramisection for the relief of spastic paralysis in human limbs has been advocated in the belief that these nerves exist and are responsible for that condition.

Recent investigations have gone to show that if the operation be sufficiently carefully performed removal of all its sympathetic nerves has surprisingly little effect on the musculature of the mammalian limb. Its posture and reflex responses are absolutely unaffected. There seems, however, from the recent investigations of Maibach (9) to be good evidence that the effect of these nerves upon muscle fatigue, first described by Orbeli (10), is valid. But it is not impossible that this may ultimately be found to be a humoral effect, and due to the action of vascular nerves; as an analogy one could point to the recent tendency to ascribe the pseudomotor responses of de-efferented muscle (Vulpian phenomenon) to the excitation of the sensitized muscle by the acetylcholine liberated by vaso-dilator nerves (Dale and Gaddum, 4).[1] In short, it did not seem impossible that a renewed attempt to demonstrate

[1]Gedewani (1930), working in Beritoff's laboratory at Tiflis, has offered a purely vascular interpretation for the Orbeli phenomenon (Jl. de med. exp. et de biol., Moscow, 13, p. 28).

sympathetic endings on muscle fibres might give negative results, and in the present research negative results have been obtained.

In this paper we refer only to such work as has been done specifically on the mammalian limb muscles and where the method of experimentally isolating the sympathetic nerves by appropriate degeneration experiments has been used. There is no other way by which we can with certainty demonstrate the sympathetic or somatic origin of a particular nerve fibre, for Langley (8) found that many postganglionic sympathetic fibres could be weakly medullated, and that some nerve fibres that emerged through the ventral roots might be only very weakly medullated or even become non-medullated.

The method adopted is that of severing the nerves to the muscles concerned proximal to the junction with the gray rami communicantes. After sufficient time has elapsed for complete degeneration of all somatic fibres the muscle is examined for intact nerve endings, which must of necessity be of sympathetic origin.

It might seem at first sight that positive results must carry much greater weight than negative ones, for the staining of sympathetic nerves is a notoriously unreliable matter. But positive results may also be subject to error. They depend for their validity on the assurance with which one can assert that all nerve endings that persist after degeneration has occurred can have no other origin than in sympathetic ganglia. But there are several possible sources of error: (1) Sufficient time must elapse for all severed nerves to degenerate before the animal is killed. Unmedullated fibres usually degenerate more slowly than medullated fibres; according to Boeke (3) sympathetic fibres may resist degeneration for as long as fourteen days after section, though eventually they, too, disappear. It is doubtful, therefore, whether the short period of five days allowed by Agduhr would suffice to ensure degeneration of the finer nerves that could be confused with sympathetic fibres. (2) On the other hand, if too long a period be allowed before the animal is killed, regeneration of fibres may be well in progress, and such regenerating fibres may closely resemble sympathetic fibres. (3) The frequent occurrence of pre-fixed and post-fixed plexuses (see Sherrington (11) and Langley (8)) may prove a complicating factor in isolating sympathetic fibres.

It may not be out of place to refer also to certain technical difficulties that may prove a source of error. The operation of *completely* severing the spinal nerves to the limb, especially the fore-limb, is by no means a simple procedure, and the microscopic examination of the tissue offers ample opportunity for incorrect observation. In Bielschowsky preparations the endings of sympathetic fibres usually appear as minute loops. This appearance, however, is simulated where the fine fibres have been severed with the microtome knife at a varicosity. Varicosities are often present at frequent intervals along the length of sympathetic nerves; it may often be extremely difficult to decide

whether a true ending is being viewed. That this difficulty is real is shown by the frequent statement of authors that these fine nerves *seem* to end on the muscle fibres. It is apparent from such remarks that the authors were not very sure of their observation. Nevertheless, certainty in these matters is imperative.

PERSONAL OBSERVATIONS.

For material we have used the musculature of the adult dog's foot, preferably the hind foot, since the complete division of all nerves is a simpler surgical procedure in the case of the hind limb.

The operation consisted in opening the spinal canal in the sacral region so as to expose the cauda equina. All the sacral and the last two lumbar nerves were severed proximal to the sensory ganglia. An interval of 8½ days was allowed for degeneration. All persisting nerves must therefore be either sympathetic or somatic sensory. Had any fine nerves been found terminating on muscle fibres it would not have been possible by our procedure to exclude a possible origin from dorsal root ganglia. However, the difficulty did not arise. After 8½ days' degeneration the animal was killed, the limb bled, washed with warm saline injected through the femoral artery, then fixed in 10% formalin injected through the artery, and finally preserved in formalin. As the amount of material yielded by this one animal far exceeded our needs, we did not think it necessary to sacrifice more.

From this material preparations were made: (1) after several days' fixation, by the recently introduced Bielschowsky-Gros method; (2) after about three weeks' fixation by Agduhr's modification of Bielschowsky's method; (3) at various times of fixation, from a month up to about 18 months, by the ordinary Bielschowsky process. Methods (2) and (3) have the advantage that serial microtome sections can be used. The preparations were very succesful, for this material responds admirably to Bielschowsky treatment—by successful preparations we mean those in which the sympathetic nerves, not only on large but also on small blood vessels, appear with great clearness.

The success of the operation was at once demonstrated by observing the small nerve bundles within the muscle. They consist of masses of debris of the degenerated motor nerves, traversed by numerous fine unmedullated nerves, as well as a few very coarse sensory fibres that can be traced to the muscle spindles or to the tendon organs of Golgi that usually lie close beside the spindles. Some time was devoted uninterruptedly to examining large quantities of this tissue, in the hope of finding intact nerve endings on the muscle fibres. But although the fine unmedullated nerves on blood vessels can be seen in large numbers in every preparation, we have not been able to find any instances of these nerves ending on the muscle tissue. Moreover, almost all the surviving fine nerves, once they have emerged from the nerve bundles, are

in obvious association with blood vessels, and no evidence could be found for the existence of a plexus of sympathetic fibres independent of the blood vessels.

For these reasons we must admit our inability to confirm the statement of Agduhr and of Kuntz that the muscles of the foot are innervated by the sympathetic system. If the illustrations of these authors be compared, it is difficult to see in Kuntz's work a confirmation of that of Agduhr. Most of the end-organs shown by Agduhr are surprisingly coarse, so coarse, in fact, that we could hardly have failed to find them in our material had they been present. On the other hand, Kuntz's illustrations show fine sympathetic nerves apparently ending on muscle fibres by one or two minute loops, scarcely comparable with the large end-organs pictured by Agduhr. Moreover, Kuntz states that in his material the sympathetic fibres that end on muscle accompany the blood vessels. Agduhr finds, on the contrary, that in his material the nerves often, though not always, lie close to coarse medullated fibres. It seems very improbable that these contradictory results are to be ascribed merely to the different animals employed in their experiments (Agduhr used cats; Kuntz worked with dogs). But be the explanation what it may, we must admit our inability to confirm either of these conflicting statements. We find the sympathetic nerves present in large numbers in our preparations, but almost without exception they are directly associated with blood vessels. The few apparently exceptional cases are probably due to an association with some unstained capillary.

According to Agduhr there are definite features by which we can distinguish the so-called sympathetic endings on muscle fibres from the ordinary motor endings—their "simple neurofibrillar structure, the superficial position of the neurofibrils, and especially the particularly clear impregnation of the end-loops, together with the remarkably small number of cell nuclei that exist in these plates"; the origin of fine unmedullated fibres as collaterals from coarser medullated fibres he thought improbable.

This has not been our experience in examining normal unoperated dog-muscle. In such tissue fine nerves forming small end-plates on muscle fibres undoubtedly do occur, though not in abundance. In face of the foregoing results it does not seem that they can be of sympathetic origin. To examine this point we have isolated in a dog the somatic nerves to the fore-foot by removing the stellate ganglion;[2] 38 days were allowed for degeneration. The

[2] As the operation of removal of the stellate ganglion presented some difficulty, the procedure adopted is here given. Under chloroform and ether, with the dog lying on its left side, a curved incision is made, with the convexity cranially, along the superior border of the scapula. The skin is divided, exposing the levator scapulae and the posterior serrate muscles. These are divided and the posterior aspect of the first rib cleared as far as the transverse process of the first thoracic vertebra. A portion of the first rib is then resected after separating the pleura from it. The third trunk of the brachial plexus, identified as it passes over the first rib, is gently retracted, bringing into view the rami communicantes with the stellate ganglion. By pressing away the pleura from the first and second rib necks

tissue was then prepared by the Bielschowsky method. Agduhr stated that after removing the stellate ganglion, nerve terminals possessing the above described "sympathetic" characteristics disappear. This statement seems very surprising, for some of the nerve terminals he figures one would never have suspected of arising from sympathetic ganglia, except for the statement that all somatic nerves had been destroyed (e.g., figure 7 of Agduhr's paper). We find in our preparations that these finer nerve endings, which are not very common in normal unoperated tissue, still persist after the stellate ganglion has been removed. Examples are shown in figures 1–4.

Figure 1 shows a typical coarse motor end-organ. From the nerve fibre arises also a small delicate collateral, terminating by a single end-loop to form an end-organ outside the main end-plate. In this subsidiary sole-plate only two nuclei are visible. It has all the characteristics of Agduhr's "sympathetic" endings, but is nevertheless a somatic motor nerve. In figure 2 is shown an example of a coarse nerve fibre giving rise to a fine collateral that ends on the muscle fibre by a simple loop. In this case a sole-plate nucleus could not be seen, though there was present a telolemmal nucleus (not shown in the figure), lying immediately above the ending. In figure 3 two very fine fibres arise from a slender medullated (?) nerve, and end by minute loops in the sole-plate of a muscle fibre; in addition a very delicate, evidently non-medullated fibre ends in the same sole-plate, but it was not possible to trace it further back in this or the preceding section. Such endings are very rarely found in this or in unoperated material, and are reminiscent of some of the end-organs discovered by Boeke (2) in very young animals. In figure 4 is shown a very coarse motor nerve terminating by a typical branching end-ramification in a large multinucleate sole-plate; within the same sole-plate there terminates by a minute loop a thin non-medullated fibre. In the previous section this fibre can be traced back a considerable distance; it thickens as we trace it backwards, but as far as it is possible to follow it it remains independent of the coarse medullated fibre. Even in normal tissue such an "accessory" ending is very rarely seen. Its sympathetic origin is excluded because the stellate ganglion has been removed. It seems probable that the condition is a vestige of those remarkable cases of multiple innervation of single sole-plates that Boeke has discovered to be very common in embryonic muscle tissue.

Our conclusion that sympathetic fibres do no innervate the striated muscle

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the sympathetic trunk is exposed; it is retracted with a small hook and divided distal to the stellate ganglion. The latter is then seized with forceps, its connection with the brachial plexus severed with fine scissors, taking care not to pull on the plexus, and the whole ganglion removed. The divided muscles are then again sutured with catgut and the skin apposed with silk-worm sutures.

The sympathetic nature of the ganglion was subsequently identified by histological means; success of the operation was further confirmed by the following effects: increased warmth of right foot, engorgement of right conjunctiva, narrowing of palpebral fissure, contraction of pupil.

fibres of the foot must be made with one reservation. In the absence of physiological evidence for a direct supply of sympathetic nerves to muscle there would be no reason to suspect it. But the Orbeli effect, as Maibach's recent

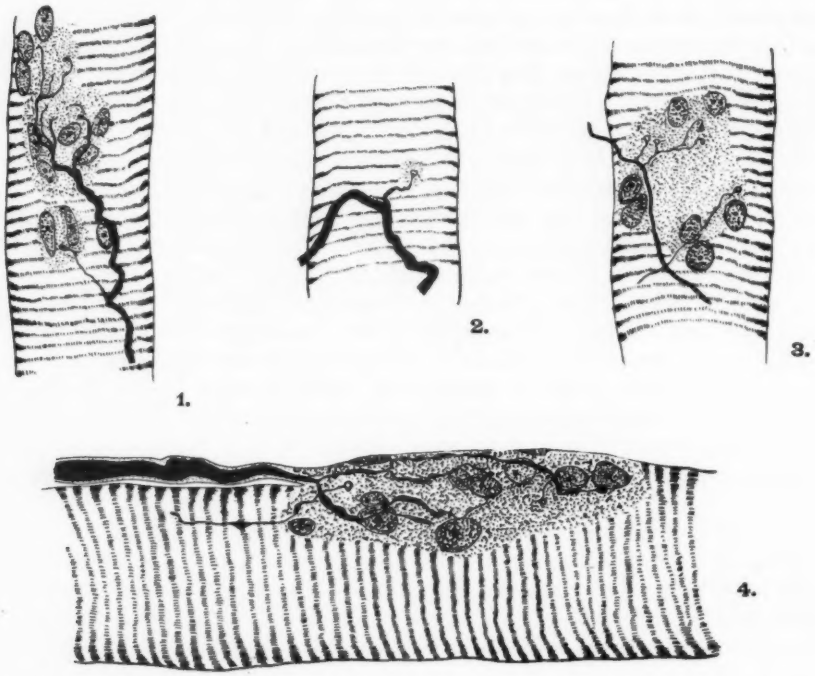

Figure 1. Coarse medullated nerve fibre ending by typical branching motor end-organ; from the same nerve fibre arises a delicate collateral which ends by a simple loop outside the main "end-plate", and on the same muscle fibre.

Interosseus muscle of adult dog; stellate ganglion removed 38 days; Bielschowsky preparation.

Figure 2. Coarse medullated fibre giving rise to a fine collateral, that ends in a single minute loop.

Interosseus muscle of adult dog; stellate ganglion removed 38 days; Bielschowsky method.

Figure 3. Slender medullated (?) fibre giving rise to fine collaterals that end in several minute loops in a large mass of nucleated sole-plate protoplasm; a second non-medullated fibre ends in the same sole-plate.

Interosseus muscle of adult dog; stellate ganglion removed 38 days; Bielschowsky method.

Figure 4. Ending of coarse medullated fibre by a typical branching end-ramification. Within the sole-plate protoplasm there also terminates by a minute loop a thin non-medullated fibre. As far as it is possible to trace this fibre backwards in this and the previous section, it remain quite independent of the coarse fibre.

Interosseus muscle of adult dog; stellate ganglion removed 38 days; Bielschowsky method.

work shows, seems to be an established phenomenon. As already remarked, we could perhaps account for this effect along the lines advocated by R. Heidenhain, Gasser, Dale, and others for the explanation of the contracture of de-efferented muscle when dorsal root fibres are stimulated, namely, by the liberation of a vaso-motor substance (acetylcholine of Dale) from the endings of vaso-motor nerves. Nevertheless, the existence of fine filamentous collaterals, unstainable by present methods, arising from the sympathetic nerves on blood-vessels, and ending in the striated muscles fibres, cannot thereby be excluded. No histological methods have yet succeeded in displaying fine filaments which terminate within the plain muscle fibres of arterioles; if such occur, it is not impossible that similar connections also exist with the striated muscle fibres. Nevertheless, in our preparations, we never found an indication of their existence.

The Muscle Spindles. The fibres of the muscle spindles possess, as is well known, both a motor and a sensory innervation. Agduhr believes that they are innervated also from the sympathetic system. Hines and Tower (5), and also Hinsey (6), were able to observe only the sensory and motor innervation.

The sensory nerves are usually applied to the muscle fibres of the spindle in spirals wound round and round the fibres, and come into intimate association with them; the motor endings are usually small *terminaisons en grappes*, situated at the end of the spindle.

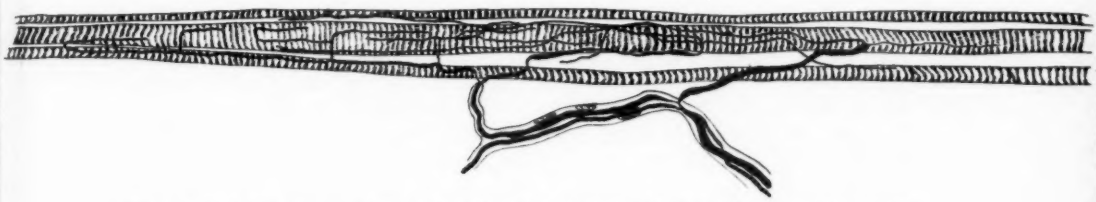

Figure 5. An uncommon form of ending of a sensory fibre within a muscle spindle. The coarse medullated fibre breaks up into long delicate filaments, closely resembling sympathetic nerves, and applied to the surface of the spindle fibres. Similar endings persist after degeneration of all motor fibres. Foot muscle of adult dog; stellate ganglion removed 38 days; Bielschowsky preparation.

In our preparations of the foot muscles of the dog, in which all somatic motor nerves have degenerated 8½ days, the somatic sensory fibres are always to be seen; but although sympathetic nerves are visible on neighbouring blood vessels in large numbers, we never find them within the spindles. We cannot but conclude that Agduhr must have employed incorrectly operated material, or tissue from which the somatic nerves have not completely degenerated.

In support of this we find that the somatic sensory endings in the spindles

of the foot muscles do not always terminate in thick cords wound spirally around the spindle fibres, but they may, on the contrary, develop into long, delicate fibrils, very closely resembling sympathetic nerves, and running for long distances along the length of the spindle fibres (figure 5). Their sympathetic origin is excluded by the fact that they arise from very coarse medullated fibres, and by the fact also that they occur in the dog even when the stellate ganglion has been removed. Their motor origin is excluded by the fact that they occur in the dog from which all motor nerves have degenerated 8½ days.

SUMMARY.

1. In the muscles of the dog's foot, when all somatic motor nerves have completely degenerated, leaving sensory and sympathetic fibres uninjured, the only persisting intact nerves are certain coarse somatic sensory fibres and the nerves of blood-vessels. No evidence could be found for a direct sympathetic supply to the striated muscle fibres.

2. When sympathetic nerves are destroyed, nerve-endings persist on the muscle fibres similar to those to which a sympathetic origin has been ascribed.

We wish to express our appreciation to Professor MacCallum for the facilities given us for the surgical work.

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"ROSE WILT" AND "DIEBACK." A VIRUS DISEASE OF ROSES OCCURRING IN AUSTRALIA

by

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INTRODUCTION.

The study of a rose disease, known in Australia as "Rose Wilt" and "Dieback," was commenced in March, 1929, on the representations of the National Rose Society of Australia. The disease had been very prevalent in Victorian rose gardens during 1927 and 1928, and was causing rosarians a great deal of concern. Nothing definite was known concerning the cause of the disease, though it was reputed to be bacterial in origin. All control measures tried had been found unsuccessful. The following investigations were carried out during 1929 and 1930, and the result of the work has definitely placed the disease in the class of virus diseases.

The investigations were carried out in the Botanical Laboratories in the University of Melbourne under the direction of Dr. McLennan, to whom the writer desires to express his thanks. The writer also wishes to thank Professor Ewart for his interest in the work, and for his providing the writer with the facilities wherewith to carry out the research.

HISTORY OF THE DISEASE.

The disease began to manifest itself strikingly in Victorian rose gardens during the years 1908 to 1912. It was not regarded as a very serious disease by rose growers until 1915, by when it had reached serious proportions in Victoria.

Diseased plants were examined by Brittlebank, then Government pathologist, who, according to Pescott (4), "determined the presence of an unknown bacterium in very large numbers." Pescott (5) later repeats this statement, and also avers that the bacteria had been found in the bodies of the rose aphis. Elliot (2), speaking of this disease, says that inoculations of healthy plants with bacteria gave positive results. Rossi (6), in an article in the Australian Rose Annual for 1929, refers to the disease as "Rose Wilt." He states that the

Government Pathologist made a careful analysis of plants affected with the disease, and found nothing wrong with them, but concluded that the disease was possibly caused by "some sort of bacterium." Rossi also gives an account of the disease in his book, "The Australian Rose Manual."

Elliot (2) gives the first coherent description of the disease. He says: "After winter pruning, the shoots start out in the usual way, and grow rapidly

Figure 1. Rose var. Columbia, showing recurved leaves of the Rose Wilt stage.

until from 3 inches to 1 foot in length, sometimes until the flower buds form. Then the leaves nearest the base of the new shoot curl up and drop off when touched or shaken by the wind. This continues right up the stem, the tips remaining fresh until the stem is quite bare and discoloured. Shoots break out lower down, only to share the same fate. Eventually shoots break out from the base, looking full of life and vigour for a time, but they in turn follow the others. The strong hard wood often dies back and turns black."

Brundrett (1), in a note on "Dieback of Roses" in the 1929 Australian Rose Annual, also briefly describes the disease. He states: "The symptoms of this disease are curling of the young leaves, which fall if touched, or if the tree receives a slight shake. After the leaves fall the young wood starts to die back, and it goes down through the previous years' wood, and sometimes kills the plant." Brundrett also notes the fact that the plant may apparently recover and grow vigorously for another season before succumbing.

In 1928 an alarming increase in the number of plants affected was observed, and the National Rose Society of Australia established a scholarship for research on rose diseases, Rose Wilt and Dieback diseases in particular.

The results of the writer's investigation of the problem discredits the bacterial hypothesis, and shows conclusively that we are dealing here with an unusual type of virus disease.

Examination of plants in various stages of the disease, both in the field and in the laboratory, indicate that the disease conditions known as "Rose Wilt" and "Dieback" are related, "Rose Wilt" being the primary condition of the disease in an infected rose, while the condition described as "Dieback" is due to the progress of the disease.

DISTRIBUTION.

No reference is found in the literature on rose diseases to a disease resembling this Rose Wilt condition, and so far as is known at present the disease appears to be restricted to Australia and New Zealand. In Australia the disease has been recorded from Victoria, New South Wales, South Australia, and Tasmania. In Victoria the disease first came into prominence in the eastern suburbs of Melbourne, but it has since been reported from other suburbs. In order to obtain a general idea of its distribution through Victoria, visits were made to various country centres in the State. The collection of data on its distribution is as yet far from complete.

Economic Importance.

"Rose Wilt" or "Dieback Disease" is one of the most serious diseases affecting roses in Victoria, and on account of its nature it is likely to become a limiting factor in rose-growing in Victoria. It appears generally in the form of an

epidemic, causing great damage in a season. It then appears to lose its virulence for some seasons, when it suddenly appears again.

Figure 2. Rose var. Columbia. A later stage in the progress of the disease. Some defoliation occurring.

Figure 3. Rose var. Ville de Paris. Plant almost defoliated.

SYMPTOMS.

Owing to the different appearance of affected roses at various periods, the disease is best described by dividing the symptoms up into well-defined stages or phases.

Stage 1. The first observable symptom of the disease is a curiously recurved appearance of the leaves on young shoots. The leaves sometimes have

an appearance of being bailed or crowded together on the petiole. If touched they fall off, and they are noticeably brittle in texture.

Stage 2. Defoliation commences at the tips of the stems of affected plants and works down the stem. The leaves drop off at the slightest jar, or with a puff of wind. In some cases the leaves assume a lighter green or yellowish colour before they drop off.

Stage 3. After an interval of about one day the tips of the young stems begin to discolour and die back for a distance of one to two inches. The rest of the young stem then develops a characteristic translucent yellowish-green appearance, which may be noticeable for a day. Later on, towards the base of the stem, this translucent yellowish-green appearance becomes intensified, and within a few hours this region at the base of the stem turns a brownish-black colour. Young leaf buds in the basal browned area remain green for some time. This appearance is characteristic of the disease in the field.

Stage 4. The whole of the stem gradually discolours and dies back, and this "dieback" condition may extend down into the older wood. Developing leaf buds become brown at the tips and then rot away. (Figures, 4, 5, 6).

Stage 5. In many cases the plant sends up one or more watershoots after the upper stems have died back. These, however, soon show signs of the disease, which progresses as described above.

Stage 6. After showing all or part of the symptoms of disease, as above, a plant may to all appearances recover, and continue normal growth for one or two seasons. Ultimately, however, disease symptoms show afresh, and the plant soon presents a withered and unsightly appearance.

CULTURAL STUDIES.

The investigation of this disease was first begun at the end of summer (March), and plants affected were in the "dieback" condition. The following procedure was adopted in an endeavour to isolate a causal organism:

Twigs from infected rose plants were washed with a 1 in 1,000 dilution of mercuric chloride solution, twice in sterile water, after which they were ground up in a sterile mortar with either broth or saline. The liquid, with some of the mashings, was then pipetted into a sterile test tube, and allowed to stand for one to three hours. At the end of that time lemco-peptone agar plates were poured using this liquid and dilutions of it as an inoculum. The plates were incubated at 25°C.

Both bacteria and fungi were isolated from various portions of diseased rose plants. Examination of the various cultures showed that some common organisms, such as *Alternaria*, *Verticillium*, *Macrosporium*, *Phoma*, and *Gloeosporium* were generally present in the dead portion of the rose twigs. Numerous types of bacterial colonies also appeared on the plates.

Figure 4. Rose var. Columbia. Plant defoliated and dying back.

Both fungal and bacterial types of organisms were subcultured, and inoculation experiments carried out, using healthy potted rose plants. Negative results were recorded in all cases.

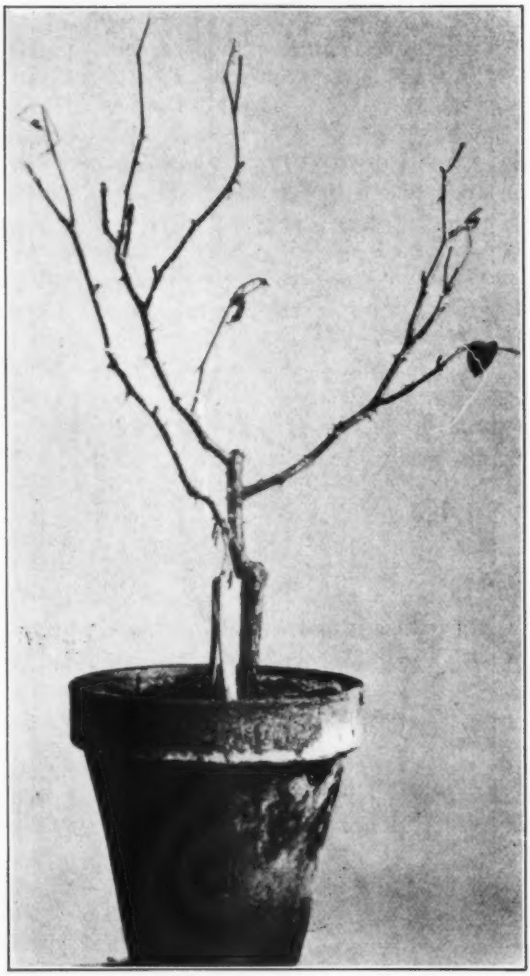

Figure 5. Rose var. Sunburst. Plant defoliated and dying back.

It was then thought that perhaps the type of media being used was unsuitable for the growth of the pathogenic organism. Accordingly various media, including rose agar decoctions, rose peptone agar, Coon's agar, and plain agar,

were used in a series of isolation experiments. Isolations were also tried using the Lemco peptone agar standardized to pH values ranging from 6·6 to 7·4.

No one type of bacterial or fungal colony, however, appeared constantly on the plates, as one would expect if the disease were bacterial or fungal in origin. Further inoculation experiments were entirely unsuccessful.

Isolation Experiments with plants showing the early stages of the disease.

In the spring of the succeeding year rose plants showing the early stages of the disease were obtained. The material was treated as described above, but examination of poured plates from numerous diseased rose plants showed no constant development of any one type of bacterial or fungal colony. Inoculation experiments with some colonies which came up on the plates were unsuccessful.

In some of the experiments, where diseased plants in an early stage were used, the plates remained sterile, although media of various kinds were again used.

Examination of Diseased Tissues.

Leaves and twigs of affected roses, both in the early and in the "dieback" stage of the disease, were fixed in Flemming's, Bouin's, and Carnoy's fixatives, and after embedding in paraffin sections were cut at various thicknesses from 5–10μ. Leaves and stems of healthy rose plants were also fixed, and sections cut from 5–10μ in thickness. Heidenhain's iron alum haemotoxylin was the stain mainly used, the counter-stain being eosin.

Examination of transverse sections of twigs and stems of plants in the "dieback" stage of the disease showed the presence of bacteria and of fungal hyphae, but the close examination of sections of twigs and stems of rose plants in the "rose wilt" stage of the disease failed to disclose the presence of any organisms.

These negative results, together with the negative results obtained in numerous isolations earlier in the year, indicated the probability of the disease being caused by an ultra-microscopic organism or particle.

INOCULATION EXPERIMENTS WITH UNFILTERED AND FILTERED JUICE.

As a preliminary test, to see whether there was an infectious principle in the juice, inoculation experiments were carried out using unfiltered juice from a diseased rose plant. The inoculated plants were kept in the laboratory. Symptoms of the disease began to manifest themselves by the end of the second week. Results are given in tabular form below.

Plants inoculated.	Inoculum.	Date of inoculation.	Date of appearance of symptoms.
Rose var. Sunburst	Unfiltered juice from an infected rose	5.10.29	15.10.29. Primary symptoms showing; leaves recurved and brittle. 20.10.29. Defoliation commencing. 28.10.29. Plant defoliated. 1.11.29. Dieback condition commencing.
Rose var. Sunburst	Juice from a healthy rose plant	5.10.29	1.11.29. Control still healthy.
Rose var. Mrs. McKee	Unfiltered juice from an infected rose	5.10.29	25.10.29. Plant showing recurved appearance of leaves. 31.10.29. Flower bud fell off before opening; defoliation commencing.
Rose var. Mrs. McKee	Juice from a healthy rose plant	5.10.29	31.10.29. Control healthy.

These results showed that there was an infectious principle in the expressed juice of diseased roses.

Experiments were next carried out to see whether the infectious principle could pass through a Seitz filter. The material was obtained from rose plants showing the early stages of the disease, and the expressed juice and mashings were diluted in broth before filtration. The filter used in this series of experiments was a Seitz E. K. Schichten type, Grosze 6. Before use the filter, with all connections, was sterilized in the autoclave at 130°C. for thirty minutes.

The juice was filtered at a pressure of 200 mm., using a vacuometer; the time of filtration was five minutes, and the volume filtered was 20 cc.

To check the filter a suspension of *B. pyocyaneus* or of *B. prodigiosus* in broth was added to some unfiltered rose juice, and this was filtered under the same conditions as above. Lemco peptone agar plates were poured from both lots of filtered juice. The reliability of the filter was proved by the fact that all the plates poured from filtered juices remained sterile. The above procedure was followed in all filtration experiments performed, using the Seitz filter.

TECHNIQUE OF INOCULATION USING FILTERED JUICE.

The following method, recommended by McKinney (3), was used in making juice inoculations. A uniform amount of absorbent cotton wool was wrapped on the point of a sterile needle. To this was added several drops of the infectious juice, using a small bacteriological pipette. The saturated cotton wool was applied to the axil of the petioles and pushed down. The leaf and petiole was scratched and pricked with the sterile needle. Controls in the following series

were scratched and pricked with sterile needles. In later experiments controls plants were inoculated with juice from healthy rose plants. The plants were kept in the laboratory close to the window.

Figure 6. Rose var. Dame E. Helen. A typically diseased rose plant. (*Photo by Porter*).

Rose var. Ville de Paris	Filtered juice from a diseased rose plant	5.11.29	8.11.29. Plant showing recurved leaves. 10.11.29. Recurved appearance of leaves more pronounced. 12-16.11.29. Defoliation occurred and the plant died back.
Rose var. Ville de Paris	Pricked and scratched with sterile needle	5.11.29	16.11.29. Control still healthy
Rose var. Golden Emblem	Filtered juice from a diseased plant	24.1.30	30.1.30. Plant showing primary symptoms of the disease. 4.1.30. Dieback condition commencing. 10.2.30. New shoots put out by the plant began to show recurved leaves. 14.2.30. Defoliation occurring. 16.2.30. Dieback condition commencing in the newer growth; young shoots also coming again. 27.2.30. New shoots wilting. 30.2.30. Plant died right back, and apparently dead.
Rose var. Golden Emblem	Scratched with a sterile needle	24.1.30	3.3.30. Plant still healthy.
Rose var. Columbia	Filtered juice from a diseased rose	3.2.30	20.2.30. Rose wilt stage commencing. 22.2.30. Dieback condition initiated. 26.2.30. Young growth showing. 10.3.30. Young growth diseased and plant dying back. 12.3.30. Plant killed right back.
Rose var. Columbia	Scratched with sterile needle	3.2.30	12.3.30. Plant still healthy.

Experimental Inoculation of Roses Growing in the Open.

Several experiments were carried out with rose plants growing in the open, but up to the time when the work was interrupted, owing to the writer's departure for England, no convincing examples of infection were obtained. It is possible that the disease is only dormant in these plants, and that in the following spring they will show symptoms of the disease.

Plants inoculated with filtered juice from a diseased rose.	Date of Inoculation.	Results.
Rose var. Golden Emblem	15.11.29	20.6.30. Plant still healthy.
Rose var. Frau Karl Druschki	16.11.29	3.4.30. Plant dying back and becoming defoliated; leaves, however, did not show characteristic recurved appearance. 20.6.30. No further change.
Rose var. Angele Pernet	17.3.30	20.6.30. Plant still healthy.
Rose var. Golden Emblem	17.3.30	24.3.30. Young leaves showing conspicuously recurved appearance. 31.4.30. Leaves returning to normal; no defoliation occurring. 20.6.30. Plant still apparently quite normal.
Rose var. Frau Karl Druschki	17.3.30	20.6.30. Plant still healthy.
Rose var. Golden Emblem	8.4.30	20.6.30. Plant still healthy.
Rose var. Dame E. Helen	8.4.30	20.6.30. Plant still healthy.

Control roses kept during the above experiments were still healthy when last examined (20.6.30).

Preliminary Experiments on Insect Transmission of the Disease.

The disease in the gardens and nurseries has not been thought to be connected with aphides, because the aphis is present on the roses for the greater part of the year and every year, while the disease assumes epidemic proportions only once or twice in periods varying from two to six years. It was thought, however, that the aphis might possibly act as a transmitter in periods when the disease was in evidence, and the following preliminary experiments were initiated to see if infection could be obtained by allowing aphides from diseased plants to feed on healthy plants.

Technique. Aphides from roses known to be healthy were placed on potted rose plants in which the disease had been produced by juice inoculations. They were allowed to feed on these for times varying from four to ten days before they were transferred to healthy plants. The aphides were removed from the infected plant, using a camel hair brush, and placed on the leaf of a healthy plant.

Varying numbers of adult aphides were transferred in different experiments, the numbers varying from four to ten aphides. A large lamp glass, the top of which was closed with cheesecloth, was placed over the entire plant. The lower rim of this glass chimney afforded support when pushed into the soil, and the cheesecloth over the top rendered the whole insect-proof.

Another method was to fit a small lamp glass over the shoot on which the aphides were placed, and fix it in position by means of a clamp and stand.

The outer end of the lamp glass was covered with a fine-meshed cheesecloth, held in place by a rubber band; the inner end of the lamp glass was stopped with cotton wool.

Date.	Source of Aphides.	No. of Aphides placed on each plant.	No. of plants inoculated.	Results.
22.3.30	Rose plant affected with Rose Wilt and Die-back disease.	4	2 (Golden Emblem)	20.6.30. Plant still healthy.
22.3.30	Healthy rose plant	4	1 (Golden Emblem)	20.6.30. Plant still healthy.
26.3.30	Diseased rose plant	8	1 (Etoile de Holland)	18.4.30. Plant showing disease symptoms. 30.4.30. Tips of shoots beginning to die back.
26.3.30	Healthy rose plant	8	1 (Etoile de Holland)	20.6.30. Plant still healthy.
4.4.30	Diseased rose plant	10	1 (Madame A. Chatenay)	20.6.30. Plant still healthy.

From the table above it may be seen that in only one case was a positive result recorded. From these experiments, therefore, it is not possible to say definitely that the rose aphis is an insect vector in connection with the disease.

SUMMARY.

1. An account is given of a disease of roses known as "Rose Wilt" or Die-back," occurring in Australia.

2. The symptoms of the disease may be summarized as follows:

(a) Leaves recurved on petioles, giving a wilted appearance to the plant. (Rose Wilt stage.)

(b) The leaves become very brittle and defoliation soon occurs. The twigs and branches rapidly die back, due to the progress of the disease. (Dieback stage.)

3. In the early stage of the disease (a) bacteria were not seen in sections of the diseased tissues. In the later stage (b) they were present.

4. The disease has been shown to be due to a filterable organism or particle, thus placing it in the class of virus diseases.

Transmission of the disease has been accomplished by inoculation of filtered juice. Experiments to test whether the rose aphis was an insect vector in connection with the disease yielded inconclusive results. Further work on this disease has been interrupted by the writer's departure for England.

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THE SEROLOGICAL AND BLOOD RELATIONSHIPS OF SOME COMMON AUSTRALIAN SNAKES

by

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The classical researches of Nuttall (12) showed that it is possible to differentiate members of the classes, orders, and even families, of the animal kingdom by the precipitin reactions of their sera. Strong anti-sera gave crossed reactions with heterologous sera, and these non-specific reactions were stronger with species which were closely related zoologically than with more distantly related species. Graham Smith (5) extended Nuttall's work, which was mainly concerned with mammalian and avian sera, enlarging his results by observations on the sera of reptiles, amphibians, fish, and crustaceans. Of special interest in connection with our present study are this last worker's results with rabbit anti-sera, prepared against the sera of *Tropidonotus natrix* and of *Python moluras*. With the first, good reactions were obtained with the sera of nearly half the Ophidians tested, and feeble ones with those of Chelonia, Crocodilia, and Lacertilia. The second anti-serum was weak, and reacted with the blood of only four snakes and one chelonian.

The object of the present studies was to ascertain whether serological or blood relationships could be utilized to confirm morphological findings in the differentiation of species among the venomous snakes of this country. We have therefore explored the degree of specificity exhibited in precipitin and complement fixation reactions by the sera of some of these closely allied ophidians, though it seemed unlikely that the former, even with the development of an adsorption technique, would prove of much value, particularly in view of the results of many modern workers with mammalian sera. Furthermore, the non-specificity exhibited in precipitin reactions by the venoms of poisonous snakes (Lamb, 8; Flexner and Noguchi, 4; Houssay and Negrete, 6) was not encouraging. Finally, we have utilized suitably adsorbed haemagglutinating sera, which promise a greater degree of specificity, as is indicated by the results obtained with mammalian sera by Landsteiner and Reich (9), Von Dungern and Hirschfeld (2), Morgenroth and Bieling (11), and by Landsteiner and Van der Scheer (10).

(1) *The Specificity of the Precipitin and Complement Fixation Reactions.*

Sera were obtained from snakes by bleeding them out aseptically under full ether anaesthesia through a cannula inserted into the left aortic arch. The heart and great vessels were exposed by cutting through several ventral plates, and the skin was reflected and pinned out on either side. The head was held either by hand or by a non-slip tie round the neck. During the bleeding the head and tail were elevated so as to keep the heart as full as possible. As is well known, reptilian blood, particularly when collected under such conditions, clots only with difficulty. The fluid which exudes from the clot may again clot, and a third, and even a fourth, partial clotting may occur. Some of the sera were particularly troublesome, and we used oxalate plasma instead of serum for the immunization of rabbits, though not for the precipitin tests themselves.

For the preparation of anti-sera, rabbits were used, the injections of serum (or in some of the later experiments, of oxalate plasma) being made twice weekly. In our first series we injected only small doses of reptilian sera on account of their presumably high toxicity. For example, our first anti-copperhead serum was made by injecting into a rabbit 0·5 cc. of copperhead serum subcutaneously, followed four days later by an intravenous injection of 0·2 cc., and then by four injections of 0·5 cc. twice weekly. In our later series of rabbits we gave an initial intravenous dose of 0·5 cc. of snake serum, and then four injections (two per week), each of 1·0 cc. intravenously. A few rabbits received a longer series of these injections. The rabbits were bled out aseptically eight days after the last injection.

Antisera were prepared against the sera of *Python spilotes* var. *variegatus* (the carpet snake), *Pseudechis porphyriacus* (the common black snake), *Pseudechis australis* (a common species in Northern Queensland), *Notechis scutatus* (the tiger snake), *Denisonia superba* (the Australian copperhead), *Demansia textilis* (the common brown snake), and *Acanthophis antarcticus* (the death adder). The antisera were tested against all the above-named sera, and in addition against those of *Dendrophis punctulatus* (the green tree snake) and *Denisonia maculata* (the ornamental snake). In the whole set of experiments the following genera were tested: *Python*, *Acanthophis*, *Notechis*, *Denisonia* (two species), *Demansia*, and *Pseudechis* (two species). The single python belongs to the family Boidae, and the remaining snakes to the family Colubridae. One snake, *Dendrophis punctulatus* is *aglyphous*, and the remainder are *proteroglyphous*. Boulenger (1896) places these proteroglyphous elapine genera in the following order: *Demansia*, *Pseudechis*, *Denisonia*, *Notechis*, and *Acanthophis*, and Hamilton Fairley's (3) studies of the osteological characters and biting mechanism of species in these genera confirms this order.

The antisera from the first series of rabbits were of relatively low titre; added undiluted to dilutions of snake serum 1 in 50 to 1 in 100, they immediately

showed an opalescent ring at the plane of contact of the two liquids. Macroscopic flocculation was visible after 30 minutes in a water bath at 37°C. In higher dilutions only an opalescent ring could be seen after 3 hours at 37°C.

The later series of immune rabbits gave much higher titres, and precipitation occurred immediately with dilutions of snake sera 1 in 200 to 1 in 800. In the tests reported here dilutions of snake sera were put up with an equal volume of antisera. The volume of the system was 0·2 cc.; 0·1 cc. of diluted snake serum and 0·1 cc. of antiserum. The tubes were placed in the water bath for 3 hours at 37°C. The results given in Table 1 represent the limiting dilutions of snake sera with which flocculation was visible without the use of a lens after 3 hours at 37°C.

In the complement fixation tests sheep red blood corpuscles in 3% suspension and rabbit anti-sheep haemolysin were used. Complement was obtained from guinea-pigs killed overnight. The snake serum antisera, after being diluted, were heated for 15 minutes at 56°C., and the snake sera were diluted but not heated. The number of minimal haemolytic doses of complement fixed by the antisera with various sera are given in Tables 2 and 3. In Table 2 the antisera were used in 1 in 10 dilution, except the second death adder serum, which was used in 1 in 5 dilution. The snake sera were tested in a dilution of 1 in 1,000.

In Table 3 the antisera were tested in a dilution of 1 in 10 against snake sera diluted 1 in 20,000, and in a dilution of 1 in 20 against snake sera diluted 1 in 10,000.

The results of both sets of tests, given in Tables 1, 2, and 3, show that neither method in this simple form permits of the differentiation of closely allied genera and species. In the complement fixation tests the serum of the single member of the family Boidae is clearly differentiated from all the other sera tested.

In Table 1 it is of interest to note that the antiserum prepared against the serum of *Demansia textilis* reacts as strongly in the precipitin tests with the sera of *Pseudechis australis*, *Denisonia maculata*, and *Denisonia superba*, as against the homologous serum; that antiserum (ii) against the serum of *Acanthophis antarcticus* reacts as strongly with the sera of *Pseudechis porphyriacus* and *Denisonia maculata* as against its homologous serum; that antiserum (ii) against the serum of *Denisonia superba* reacts more strongly against the serum of *Pseudechis porphyriacus* than against the homologous serum; that antiserum (ii) against *Notechis scutatus* reacts as strongly with the serum of *Pseudechis australis* as against the homologous serum; and finally that antiserum (ii) against *Pseudechis porphyriacus* reacts as strongly against the serum of *Pseudechis australis* as against the homologous serum. These results illustrate on a small scale the fact, demonstrated by Nuttall and Graham Smith, that the stronger antisera exhibit reactions with a wider range of heterologous sera.

TABLE 1.

Limiting Dilutions of Antisera which give Precipitin Test with Ophidian Sera.

Sera used for testing.	Antisera against ophidian sera.												
	Python spilotes.	*Demansia textilis.*	*Pseudechis porphyriacus.*		*Pseudechis australis.*	*Denisonia superba.*		*Notechis scutatus.*		*Acanthophis antarcticus.*		
			(i)	(ii)		(i)	(ii)	(i)	(ii)	(i)	(ii)	(iii)
Python spilotes.	1 in 6400	1 in 2000	0	1 in 2000	0	0	1 in 2000	0	0	0	0	0
Dendrophis punctulatus.	0	1 in 8000	—	1 in 4000	0	—	1 in 4000	—	1 in 100	—	1 in 2000	1 in 400
Demansia textilis.	0	1 in 16000	—	1 in 8000	1 in 400	—	1 in 8000	—	1 in 1600	—	1 in 4000	1 in 1600
Pseudechis porphyriacus.	0	1 in 8000	1 in 6400	1 in 16000	1 in 400	1 in 800	1 in 32000	1 in 800	1 in 1600	1 in 400	1 in 8000	1 in 1600
Pseudechis australis.	0	1 in 16000	—	1 in 16000	1 in 6400	—	1 in 8000	—	1 in 3200	—	1 in 4000	1 in 1600
Denisonia superba.	0	1 in 16000	1 in 3200	1 in 8000	1 in 400	1 in 6400	1 in 16000	1 in 1600	1 in 800	1 in 200	1 in 4000	1 in 800
Denisonia maculata.	0	1 in 16000	—	1 in 8000	1 in 800	—	1 in 8000	—	1 in 1600	—	1 in 8000	1 in 1600
Notechis scutatus.	0	1 in 8000	1 in 200	1 in 8000	1 in 800	1 in 100	1 in 6000	1 in 800	1 in 3200	1 in 100	1 in 6000	1 in 1600
Acanthophis antarcticus.	0	1 in 8000	1 in 1600	1 in 8000	1 in 400	1 in 800	1 in 8000	1 in 400	1 in 400	1 in 400	1 in 8000	1 in 1600

TABLE 2.

Minimal Haemolytic Doses of Complement Fixed by Ophidian Antisera (dilution 1 in 10) with Homologous and Heterologous Sera 1 in 1000.

Sera used for testing.	Antisera.						
	Python spilotes.	*Pseudechis porphyriacus.*	*Pseudechis australis.*	*Denisonia superba.*	*Notechis scutatus.*	*Acanthophis antarcticus.* (i)	*Acanthophis antarcticus.* (ii)
Python spilotes	12½	0	0	0	0	0	<1
Dendrophis punctulatus	0	0	0	2	0	0	1½
Demansia textilis	0	2	0	12	6	6	12
Pseudechis porphyriacus	0	**7**	5	10	6	6	17
Pseudechis australis	0	3½	**7**	7	2½	3½	12
Denisonia superba	0	0	0	**12**	7	2½	8
Denisonia maculata	0	2½	2½	15	8	6	30
Notechis scutatus	0	2	0	15	**12**	5	20
Acanthophis antarcticus	0	2½	0	8	6	**6**	**25**

TABLE 3.

Minimal Haemolytic Doses of Complement Fixed by Ophidian Antisera diluted 1 in 10 and 1 in 20 against Sera diluted 1 in 20,000 and 1 in 10,000.

Sera used for testing.	Antisera.							
	Demansia textilis		*Pseudechis porphyriacus*		*Denisonia superba*		*Acanthophis antarcticus*	
	1/10	1/20	1/10	1/20	1/10	1/20	1/10	1/20
Python spilotes	0	0	0	0	0	0	0	0
Dendrophis punctulatus	3	1	3	2	0	1	0	1
Demansia textilis	**18**	**20**	7	7	3	2	3	0
Pseudechis porphyriacus	6	15	**15**	**18**	2½	5	3	0
Pseudechis australis	9	10	12	12	3	3	0	0
Denisonia superba	15	15	12	15	**12**	**17**	4	0
Denisonia maculata	9	12	6	15	4	5	4	0
Notechis scutatus	9	7	7	7	3	6	3	0
Acanthophis antarcticus	7	7	10	8	3	6	**6**	**4**

The complement fixation tests are somewhat more specific. The homologous serum almost invariably fixes more complement than any of the closely allied heterologous sera, and the extent of the non-specific reactions given by these latter are generally in accord with the zoological order of the genera in the proteroglyphous division of the family Colubridae. However, in the two cases studied the results have no very decisive value in allotting a species to its genus. The sera of *P. australis* and *P. porphyriacus* in most of the tests in Table 2 appear to be more closely related to each other than to any of the other sera

tested, but the relationship between *D. superba* and *D. maculata*, judged by these tests, is less close than that between the latter and *Acanthophis antarcticus*.

The extremely close zoological relationship of *Notechis scutatus* with *Denisonia superba* is clearly indicated by the results in Table 2, but these species are sharply differentiated by those in Table 3. On the whole it seemed unlikely that complement fixation in this simple form would prove of much value for our purpose.

Despite the theoretical unsoundness of the absorption method as applied to the precipitin reaction we attempted to obtain antisera with a more specific precipitating action by this method. A number of experiments were made with the antisera against *Demansia textilis*, *Pseudechis australis*, *Pseudechis porphyriacus*, *Denisonia superba*, *Notechis scutatus*, and *Acanthophis antarcticus*, using for absorption various dilutions of those heterologous sera which gave the highest non-specific precipitation. For this purpose the snake sera were diluted 1 in 25, 1 in 50, 1 in 75, or 1 in 100, and undiluted antiserum was added in the proportion of 0·2 cc. to 1·0 cc. of the dilutions of serum. The mixtures were incubated in a water bath at 37°C. for 2 hours, and left in the ice-chest overnight. They were then centrifuged, and the supernatant fluid used for the tests.

The antiserum to *Demansia textilis* was subjected to a double absorption, using the sera of *P. porphyriacus* and *Notechis scutatus* 1 in 10. The original titre of this antiserum to its homologous serum was 1 in 16,000. This was reduced to 1 in 2,000, and the absorbed serum gave no non-specific precipitin reactions. Unfortunately no sera from other species of the genus *Demansia* were available to test the species specific value of the absorbed antiserum.

Pseudechis australis antiserum, after absorption with the serum of *Demansia textilis* 1 in 100 and 1 in 50, gave good reactions to 1 in 500 with *Pseudechis porphyriacus* serum, but exhibited a well-marked zone phenomenon with the homologous serum, reacting only with 1 in 250 and 1 in 500 dilutions. Absorbed with the serum of *Notechis scutatus* in the same dilutions, it reacted only feebly (1 in 50 and 1 in 100) with the serum of *Pseudechis porphyriacus*, and not at all with the homologous serum. This absorbed antiserum appeared to be specific for the genus but not for the species.

Pseudechis porphyriacus antiserum, absorbed with the serum of *Acanthophis antarcticus* 1 in 25, reacted only to *Pseudechis australis* 1 in 50 to 1 in 250, and to the homologous serum (1 in 50 to 1 in 500), but we were unable to obtain this result in further experiments.

Denisonia superba antiserum, when absorbed with the serum of *Pseudechis porphyriacus*, appeared to lose its activity to about the same extent against the homologous serum and against the sera of *Acanthophis antarcticus* and *Notechis scutatus*, which, like that used for absorption, gave strong reactions with the unabsorbed antiserum; but the almost equally strong reactions to the sera of

Denisonia maculata, *Pseudechis australis*, and *Demansia textilis* were extinguished, as also was the reaction to the serum of *Dendrophis punctulatus*.

Notechis scutatus antiserum absorbed with the serum of *Pseudechis porphyriacus* 1 in 25 gave on one occasion a supernatant fluid reacting only with the homologous serum 1 in 50 to 1 in 250. A double absorption with the serum of *Pseudechis porphyriacus* 1 in 25 and *Denisonia superba* 1 in 25 also in a single experiment yielded a supernatant fluid reacting only with the serum of *Notechis scutatus* 1 in 50 and 1 in 100. Two other absorptions with *P. porphyriacus* 1 in 50 and 1 in 100 gave absorbed antiserum reacting only with the homologous serum 1 in 50 to 1 in 100 and 1 in 50. Great difficulty was experienced in this, as in other cases, in repeating successful results so as to obtain a stock of absorbed serum specific for one genus, and irregular results were obtained with absorptions carried out under apparently identical conditions.

Acanthophis antarcticus antiserum was absorbed in a number of experiments with *Pseudechis porphyriacus* serum. In no case was a satisfactory result obtained, the reactivity to the homologous serum disappearing coincidentally with that to the serum used for absorption, though other non-specific reactions were practically eliminated. Absorbed with a dilution of 1 in 25 of this serum, the antiserum reacted to *P. porphyriacus* (1 in 50 to 1 in 100), to *Notechis scutatus* (1 in 50 to 1 in 250), and to the homologous serum (1 in 50 to 1 in 100).

The results of these absorption experiments were on the whole disappointing. Though it seemed possible that antisera might be obtained which would be available for the identification of the genus but not the species of a given reptile, it was obvious that a larger number of species of the proteroglyphous Colubridae would need to be investigated before such a use of the method would be justified. The irregularity of the results of absorption under apparently identical conditions led us to choose for further work the haemagglutination reaction, and we have made some preliminary investigations of the specificity of this method.

(2) *The Specificity of the Haemagglutination Reaction.*

Preparation of antisera. Snakes were bled out aseptically into an equal volume of 2% sodium citrate solution in normal saline. The red blood corpuscles were washed four times by centrifugation with normal saline and injected intravenously into rabbits.

Rabbits immunized with the cells of *Acanthophis antarcticus*, and those of *Pseudechis porphyriacus* and of *Denisonia superba*, received three intravenous injections at intervals of four days: 2·0 cc. and 2·5 cc. of 20% suspension, and 2·0 cc. of 50% suspension of the cells in normal saline.

Rabbits immunized against the red cells of *Notechis scutatus* received four injections at a similar interval of 1·0 cc., 2·0 cc., and 2·5 cc. of a 20% suspension and 2·0 cc. of a 40% suspension in normal saline. The rabbits were bled out on the sixth day after the last injection. It was found necessary to use the

cells of *Notechis scutatus* as soon as possible after bleeding out the snake, as they became toxic after being kept for three or four days.

Method of testing. The antisera were heated for 20 minutes at 56°C., and 0·1 cc. of a 2·5% suspension of four times washed red blood cells was mixed with 0·5 cc. of various dilutions of antisera. The readings were made after standing 2 hours at room temperature 25–30°C. The red blood cells of some species (copperhead, brown snake, and tiger snake) were haemolyzed to a slight extent when diluted with 0·85% saline. This difficulty, which was most evident with the weakest dilutions of antisera, was overcome by using 0·95% saline. Normal rabbit serum was used as a control, and gave no agglutination in dilutions between 1 in 10 and 1 in 320. In Table 4 absence of reaction is therefore expressed as indicating a titre of less than 1 in 10.

Technique of absorption. To 5·0 cc. of a 1 in 10 dilution of antiserum 0·5 cc. of well-centrifuged washed blood sediment was added. After thorough mixing this was allowed to stand at room temperature for 2 hours and in the ice-chest overnight. After centrifuging, the tests were made by mixing 0·5 cc. of various dilutions of the absorbed haemagglutinating serum with 0·1 cc. of 2·5% suspension of washed red blood corpuscles. The readings were made as before after 2 hours at room temperature.

In Table 4 there are recorded the extreme variations observed in a number of experiments, in which two haemagglutinating antisera for the red blood cells of the death adder, tiger snake, and black snake respectively, and one only for those of the copperhead, were employed. The results, considered quantitatively, fit the zoological order of the genera, except for the fact that the red blood cells of the death adder are agglutinated in a dilution of 1 in 75 by the haemagglutinating sera prepared against the red blood cells of the black snake, while those of more closely allied species do not agglutinate.

The single species of the genus *Demansia* (the common brown snake) was sharply differentiated in these tests, since its red blood cells were not agglutinated non-specifically by any of the haemagglutinating sera.

Another point of interest is the close relationship indicated between *Notechis scutatus* and *Denisonia superba*, which parallels the non-specificity observed in experiments on active immunity to the venoms of these two species (Kellaway, 7).

The results of absorption experiments, though fairly regular, were not very satisfactory. They also indicate the close relationship between *Notechis scutatus* and *Denisonia superba* and between *Pseudechis porphyriacus* and *Acanthophis antarcticus*. Low titre specific haemagglutinating sera, which, however, were tested against the red blood cells of only a few species, were obtained against the red blood cells of the death adder and of the tiger snake, but not against those of the copperhead.

Unfortunately none of the rarer snakes were available at the time when these

tests were carried out, and the supply of death adders also failed. For this reason we were unable to investigate fully our black snake and copperhead haemagglutinating sera.

TABLE 4.

Results of Haemagglutination Tests before and after Absorption.

Haemagglutinating antisera against red blood cells of:	Absorbed with red blood cells of:	Tested against red blood cells of: *Acanthophis antarcticus*	*Notechis scutatus*	*Denisonia superba*	*Pseudechis porphyriacus*	*Demansia textilis*
Acanthophis antarcticus	Unabsorbed	1 in 200	1 in 50–1 in 75	1 in 40	1 in 40–1 in 75	<1 in 10
	Notechis scutatus	1 in 60	<1 in 10	<1 in 10	1 in 20–1 in 40	<1 in 10
	Denisonia superba	1 in 40	<1 in 10	<1 in 10	1 in 40	<1 in 10
	Pseudechis porphyriacus	1 in 20	<1 in 10	<1 in 10	<1 in 10	<1 in 10
	Acanthophis antarcticus	<1 in 10	<1 in 10	<1 in 10	<1 in 10	<1 in 10
Notechis scutatus	Unabsorbed	1 in 150	1 in 600–1 in 800	1 in 200–1 in 300	1 in 50	<1 in 10
	Acanthophis antarcticus	<1 in 10	1 in 160	1 in 100	<1 in 10	<1 in 10
	Notechis scutatus	<1 in 10	<1 in 10	<1 in 10	<1 in 10	<1 in 10
	Denisonia superba	<1 in 10	1 in 20–1 in 50	<1 in 10	<1 in 10	<1 in 10
	Pseudechis porphyriacus	<1 in 10	1 in 160	1 in 100	<1 in 10	<1 in 10
Denisonia superba	Unabsorbed	—	1 in 320	1 in 320	1 in 20	<1 in 10
	Notechis scutatus	—	<1 in 10	<1 in 10	<1 in 10	<1 in 10
	Denisonia superba	—	<1 in 10	<1 in 10	<1 in 10	<1 in 10
	Pseudechis porphyriacus	—	1 in 320	1 in 320	<1 in 10	<1 in 10
Pseudechis porphyriacus	Unabsorbed	1 in 75	<1 in 10	<1 in 10	1 in 240–1 in 320	<1 in 10
	Pseudechis porphyriacus	<1 in 10	<1 in 10	<1 in 10	<1 in 10	<1 in 10
Normal rabbit serum.	Unabsorbed	<1 in 10	<1 in 10	<1 in 10	<1 in 10	<1 in 10

Though the number of species tested is too small for much significance to be attached to these preliminary findings, our results do not support the general consensus of opinion in regard to the higher degree of specificity exhibited in the haemagglutination reaction. The chief advantage which this reaction possesses lies in the fact that excess of the absorbing reagents can be removed, and it is therefore possible that with alcoholic extracts of red blood cells as antigen we may achieve a higher degree of specificity by the use of complement fixation. We hope to explore this possibility in a later communication.

CONCLUSIONS.

(1) The precipitin test in its simple form does not allow of the differentiation of closely allied genera in the family Colubridae. Absorbed precipitating sera can be obtained, though somewhat irregularly, which are specific for genera but do not differentiate species.

(2) Complement fixation carried out with precipitating antisera is much more specific, and the results are in accord with the zoological order of the genera studied. For obvious reasons it cannot be applied to absorbed precipitating sera.

(3) Preliminary observations on the haemagglutination reaction do not suggest that it is more highly specific than complement fixation with precipitating antisera.

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URINARY pH AND ALVEOLAR CARBON DIOXIDE TENSION CHANGES IN A CASE OF HYPERCHLORHYDRIA

by

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(Submitted for publication 12th December, 1930.)

In 1856 Bence Jones (2) recorded his observation of a steady decrease in urinary acidity following the intake of food. This change in reaction reached its maximum after some two and one-half hours. Ringstedt (17), in 1890, reported experiments to show that the stronger the acidity of the gastric secretion the greater was the alkaline change in the urine, while in cases of achlorhydria there was no such "alkaline tide."

These early observations have been confirmed by Pawlow (14), Babkin (1), Hasselbalch (8), Hubbard (10), Fiske (7), Pesopoulos (15), Endres (6), and others. Both Hasselbalch and Endres showed in addition that the hydrogen ion concentration of the urine and the alveolar carbon dioxide tension vary in the same sense, and it is now well established that with normal kidney function excretion of acid in the urine runs parallel with the alkali reserve of the plasma. In 1921 Dodds and Bennett (5), from a study of normal cases, concluded that the secretion of hydrochloric acid into the stomach was shortly followed by a rise in alveolar CO_2 to compensate for the increased bases left in the plasma. In a case of achlorhydria no alteration in alveolar CO_2 followed the meal.

Leathes (12), 1919, had already shown that the early morning "alkaline tide" in the urine was independent of breakfast, and attributable, he believed, to washing out of CO_2, owing to increasing sensitivity of the respiratory centre on passing from the sleeping to the waking state. In the same paper Leathes supported this interpretation by showing that an alkalosis was readily produced by voluntary hyperpnoea. The increase in alveolar CO_2 observed after meals by Bennett and Dodds seems to have been accepted as generally occurring, until the recent paper of Brunton and Israels (3), who failed to find a constant rise of alveolar CO_2, and criticized the contention that changes in alveolar CO_2 tension represent the adjustment of hydrogen ion concentration of the plasma following upon the secretion of free acid into the stomach.

It had never been clear to the author why secretion of gastric juice, associated as it is with simultaneous pancreatic secretion, the latter reaching its maximum rate of formation three hours after a meal, should be directly responsible for a change in urinary acidity. Moreover, it seemed doubtful whether the amount of acid secreted, even if unassociated with simultaneous pancreatic secretion, was sufficient to call for other mechanisms of hydrogen ion adjustment than the buffering provided by the blood and tissue fluids. Accurate figures for the rate of gastric secretion in the normal man are not available. Maclean and Griffiths (13) have shown that results obtained from gastric fistulae are abnormal, owing to the absence of the inhibitory effect of acid accumulation within the viscus. For the purpose of an approximate calculation only, Rosemann's (18) value of 1,000 cc. of juice for a three-hour period may be taken. This gives a value of less than 100 cc. of N/10 hydrochloric acid per 20-minute period. Assuming the effect of such a quantity to be manifested at one moment of time, and that all other mechanisms are disregarded, and the hydrogen ion concentration of the plasma is readjusted entirely by the respiratory mechanism, a rise of pressure in the alveolar CO_2 of 4·8 mm. Hg. may be expected. This figure is of the order of Dodd's results, but actually the effect would be spread over 20 minutes, and other mechanisms for compensation, such as are provided by plasma alkali, protein, corpuscles, and tissue fluid, are immediately available. This argument is fully developed by Brunton and Israels.

A paper by Kaye (11), adducing evidence in favour of a urinary "alkaline tide" following meals, showed the need for further experiment, and the opportunity was afforded by a sea voyage to England in October, 1929. The diet differed from the shore diet of the subject only in that it contained rather more protein. Urine was collected at as frequent intervals as possible, and always immediately before and immediately after each meal, from 6 a.m. to 10 p.m. inclusive. The pH was determined colorimetrically (phenol red), taking appropriate precautions against loss of CO_2. The Hellige disc comparator was employed, by which readings can be made to within 0·05 pH unit.

The alveolar carbon dioxide was estimated by Shakespeare's catharometer method, using the Knipping instrument made by Siemens and Halske. The continuous rebreathing method of Plesch (16) was adopted for obtaining samples of alveolar air. Since this method aims at equilibrating the respired air with the venous CO_2 tension, the values are higher than those obtained by the Haldane and Priestly method. The method was adopted for reasons of expediency, and did not give entirely satisfactory results in my hands. There was an inconstant zero error, and the author is by no means certain that a true equilibrium was attained in the period of 20 seconds of rebreathing. The results are recorded only because they tend to move in a more or less regular manner, and weight is not attached to the absolute values.

Data were collected whenever opportunity offered. The results are plotted

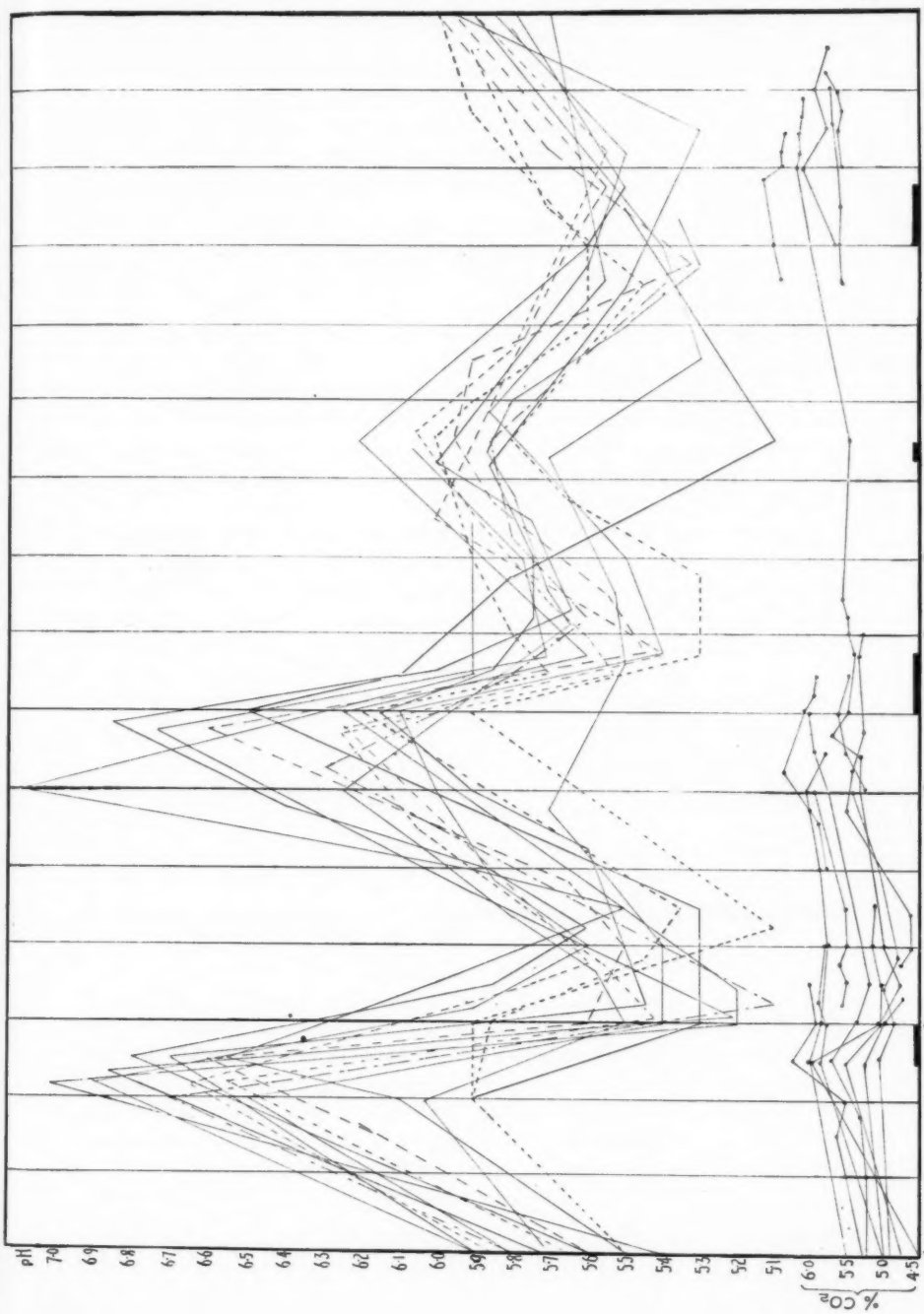

Figure 1. Complete diagram illustrating changes in pH of urine and percentage of carbon dioxide in alveolar air from 6 a.m. to 10 p.m.

graphically in figure 1. Graphic presentation is adopted in consideration for space, and because the times of collection of samples are quite irregular.

DISCUSSION.

It will be seen from the diagram that there is a well-marked morning alkaline tide, but without the fall in alveolar CO_2 tension observed in Leathes' experiments. Following the meal there is a steep rise in urinary acidity, which is followed by a return to approximately the original reaction. Campbell (4) has reported a similar transient "acid tide" in his experiments, which, like the present one, were carried out in the tropics.

The midday meal is followed by the same change in the reaction of the urine, but after afternoon tea (a cup of tea without milk or sugar, and a piece of cake) only a small increase occurred. Dinner was taken when the urinary pH was low, and following the meal there was a steady uninterrupted rise of pH until the last specimen of urine had been collected. The percentage of alveolar CO_2 ran parallel with the pH of the urine, as found by Hasselbalch and Endres.

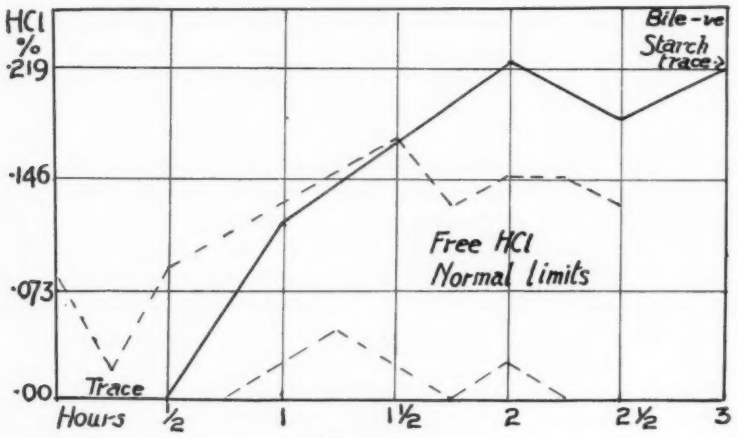

Figure 2.

It is impossible to explain the secretion of an acid urine during the early phase of gastric digestion in terms of a lowering of the hydrogen ion concentration of the blood. If the alveolar CO_2 percentages mean anything they are either a cause or a result of the varying secretion of hydrogen ions by the kidney. It is probable that my gastric secretion was not normal, for a few months later acute gastric symptoms occurred, and physical examination, fractional test meal, and X-ray examination led to the diagnosis of hypermotility with hyperchlorhydria, and hypersecretion (figure 2).

From a study of the carbon dioxide dissociation curves of the arterial and venous blood in cases of hyperchlorhydria, Hitzenberger and Tuchfeldt (9) found a subnormal pH of the plasma, and since there was no alteration of respiratory rate in these subjects, a lowered sensitivity of the respiratory centre may be the explanation. On the other hand, the careful attempt of Brunton and Israels to find evidence of alteration in sensitivity of the respiratory mechanism was not entirely successful.

In view of the evidence so far put forward, together with the results of the present observations, one can only say that the part played by the various mechanisms to adjust for the secretion of HCl into the stomach will not be clear until we know more accurately the changes of the hydrogen ion concentration in the plasma during digestion. Investigation of alveolar CO_2 tension, gastric acidity, and plasma pH is at present in progress in a series of subjects.

SUMMARY.

Observations are presented on the changes in urinary pH and alveolar (venous) CO_2 percentage in one subject (who was probably hyperchlorhydric) from 6 a.m. to 10 p.m., and show the influence of ordinary meals.

The first and second meals of the day were immediately followed by excretion of an acid urine, which became progressively more alkaline until the next meal was ingested. Afternoon tea was associated with a similar though less extensive change in urinary reaction, while the evening meal was followed by a small uninterrupted fall in acidity.

Alveolar CO_2 percentages moved parallel with urinary pH values.

The alterations in urinary pH following breakfast and luncheon in the subject studied are in the opposite sense to those which would be expected from a lowered hydrogen ion concentration in the plasma following on the secretion of gastric hydrochloric acid.

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SEX DETERMINATION IN *FRANKLINIELLA INSULARIS* FRANKLIN (THYSANOPTERA)

by

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During the course of our investigations on the thrips *Frankliniella insularis* (Franklin), the results of which were recently published (2), it was observed that eggs from unmated females developed into males only. On the other hand, both males and females were invariably obtained from eggs laid in cultures in which both sexes were present and ample apportunity for mating occurred. Since the publication of the paper referred to above, further data have been obtained from breeding experiments, from which it is now possible to interpret the relationship of sex and parthenogenesis in this species.

Parthenogenesis is known to occur with several species of Thysanoptera, but with the exception of a paper by Shull in 1917 (5), dealing with *Anthothrips verbasci*, there is no other record in Thysanoptera, so far as we are aware, in which parthenogenetic eggs develop into males only.[2] With some species in which parthenogenesis is known to occur, males may be rare or unknown, as, for example, in *Taeniothrips inconsequens* (Uzel) (1), and *Heliothrips haemorrhoidalis* (Bouché) (3). These species have been studied by many observers, owing to their importance as economic insects, so that the scarcity of males recorded is not due to paucity of observations. It would appear that in these cases parthenogenetic eggs normally develop into females only.

The species *F. insularis* evidently affords an example of arrhenotoky, and this being so, it would be expected that fertilized eggs would develop into females only, as occurs in the classic instance of the honey bee.

Breeding experiments were carried out in order to obtain data which might explain the reason why both sexes developed from eggs laid by mated females.

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[1] Mr. Bald is an officer of the Division of Plant Industry, Commonwealth Council for Scientific and Industrial Research, located at the Waite Institute in connection with investigations on Spotted Wilt disease of tomatoes.

[2] We are greatly indebted to Professor T. H. Morgan for drawing our attention to Dr. Shull's paper. Our results agree closely with those obtained by Shull.

Experiment A: Sex of individuals reared from eggs laid by virgin females of F. insularis.

Thirteen females, taken as they emerged from the pupal stage, were placed in a breeding globe on a tomato plant (a) on July 16, 1930. They were transferred to a fresh plant (b) on July 26, again transferred to a fresh plant (c) on August 7 (only 10 females alive), and again (d) on August 19. Eggs were laid in the tissues of the leaves during the periods of confinement on the successive plants. As the individuals which hatched out from these eggs became adult they were killed off and the sex recorded. The results are given in Table 1.

TABLE 1.

Showing sex of individuals reared from eggs laid by virgin females of F. insularis.

Expt. A.	Date females transferred for egg laying.	Date offspring adult.	Sex of offspring. ♂	♀
(a)	July 16-26	Aug. 18-19	50	0
		Aug. 23-27	7	0
(b)	July 26-Aug. 7	Aug. 23-26	31	0
		Aug. 26-Sept. 2	6	0
(c)	Aug. 7-19	Aug. 29-Sept. 4	52	0
(d)	Aug. 19-27 (females died)	Apparently no eggs laid, no larvae developed	–	–
		Total	146	0

Experiment B: Sex of individuals reared from eggs laid by single mated females of F. insularis.

Four newly-emerged virgin females were isolated in January, 1931, in separate breeding globes on tomato plants, and newly-emerged males introduced into each globe. The number and sex of the individuals which developed from eggs laid by these females were as follows:

(a) 23 ♀ + 7 ♂ = 76·7% ♀
(b) 18 ♀ + 17 ♂ = 51·4% ♀
(c) 14 ♀ + 6 ♂ = 70·0% ♀
(d) 14 ♀ + 12 ♂ = 53·8% ♀

Experiment C: Sex of individuals reared from eggs laid by females of F. insularis in a mixed colony of both sexes.

Fifteen females and 16 males, all newly emerged from the pupal stage, were taken from stock colonies on July 16, 17, and 18, 1930. They were placed in a breeding globe on a tomato plant (a). The conditions for mating were favourable. These adults were transferred to a new plant (b) on July 26, again on

August 7 (c), and again on August 19 (d). As the individuals which developed from the eggs laid during the periods of confinement on the successive plants became adult, they were killed off and the sex recorded. The results are shown in Table 2.

TABLE 2.

Showing Sex of individuals reared from eggs laid by females in a mixed colony of both sexes of F. insularis.

Expt. C.	Date adults transferred.	Date offspring recorded (adults).	Sex of offspring. ♀	♂	% females.
(a)	July 16-26	Aug. 22-23	3	2	60
(b)	July 26-Aug. 7	Aug. 24-27	27	13	67·5
(c)	Aug. 7-19	Aug. 29-Sept. 2	40	25	61·5
(d)	Aug. 19-29 (adults died)	Sept. 12-17	10	7	58·8
		Total	80	47	63

DISCUSSION.

It is evident from Experiment A that eggs laid by unmated females develop into males only. That eggs from mated females develop into both sexes is clear from the results obtained in Experiments B and C. In the known examples of arrhenotokous parthenogenesis, fertilized eggs develop only into females. If *F. insularis* is of this type, therefore, one can only assume that mated females lay both fertilized and unfertilized eggs, the former developing into females and the latter into males. Schrader in 1920 (4) showed that in the arrhenotokous race of *Trialeurodes vaporarium*, while fertilized eggs developed into females only, both sexes developed from eggs laid by mated females, owing to some eggs being unfertilized. Thomsen in 1927 (6), who confirmed Schrader's observations, found a similar condition in *Aleurodes proletella*.

The sex ratio in the offspring from mated females shown in Table 2 is of interest. The fact that females always predominated rules out the possibility that both sexes develop from fertilized eggs, since if the latter gave equal numbers of males and females the ratio of females obtained could not be greater than 50%, and normally would be less, since any unfertilized eggs which were laid would develop into males. It is seen in Experiments B and C that the number of females which developed from eggs laid by mated females is always greater than 50%. In Experiment B (a), (b), and (c), the males which were selected for mating developed from eggs laid by virgin females. In Experiment B (d) the males selected had developed from eggs laid by mated females. The sex ratio obtained in these experiments gives no indication that two types of males occur.

With greenhouse cultures of *A. verbasci*, Shull usually obtained a prepon-

derance of males, although analysis of random collections of the species in nature showed that females were in the majority. Shull states that the high percentage of males obtained in his cultures may have been due to conditions affecting chances of mating. Considering our results with *F. insularis*, this explanation is probably the correct one.

Thomsen, working with *Aleurodes proletella*, obtained with three cultures of mated females the following proportion of the sexes in the offspring:

(1) 49 ♀ + 27 ♂ = 64·5% ♀
(2) 47 ♀ + 19 ♂ = 71·2% ♀
(3) 72 ♀ + 41 ♂ = 63·7% ♀

The percentage of females obtained in our experiments corresponds closely with these results.

We may consider, therefore, in the absence of a study of the chromosomes in *F. insularis*, that sex is determined as in *Anthothrips verbasci* and other cases of haploid parthenogenesis, of which the honey bee is the classic example. Males develop from unfertilized eggs, and are presumably haploid; females develop from fertilized eggs, and are presumably diploid.

The chief area of distribution of *F. insularis* is the Central American region. Although it is found in the Adelaide area, it has never been recorded in epidemic numbers, such as occurs with certain other species of thrips in which parthenogenetic eggs develop into females. This latter method favours rapid multiplication. It would be of interest to know whether *F. insularis*, from the Central American region, in respect of sex determination, behaves in the same way as the species does in the Adelaide area.

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THE INFLUENCE OF TEMPERATURE ON THE INCUBATION PERIOD OF THE EGGS OF *SMINTHURUS VIRIDIS* L. (COLLEMBOLA)

by

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INTRODUCTION.

Sminthurus viridis, popularly known as the "clover springtail" or "lucerne flea," is of considerable importance in parts of Australia, owing to its occurrence in epidemic numbers during the winter season, when it feeds on a wide range of food plants and causes serious damage, particularly to lucerne and subterranean clover. In Australia it is recorded from Western Australia, South Australia, Victoria, and Tasmania.

The eggs are laid on the soil, and the results presented in this paper deal with the influence of temperature on the incubation period of the eggs when ample soil moisture is available. Factors affecting oviposition and the influence of soil moisture on the incubation of the eggs will be discussed in a later paper. The work is part of investigations which the writer is carrying out on the factors affecting the distribution and seasonal occurrence of the insect.

TECHNIQUE.

The insects were collected as required from lucerne plots at the Waite Institute by means of a sweep net, and individuals then transferred to the oviposition cages by the pipette method widely used by workers with small insects.

The oviposition cages were designed so as to give the females natural conditions for egg-laying as far as was practicable, and to ensure that a large number of egg batches, laid at definite periods, would be available. The cages employed were all of the same general type, differing only in size and details.

The first type of cage (type A) consists of a lamp globe $3\frac{1}{2}$ inches high, which fits snugly into a zinc cylinder 2 inches in diameter by $1\frac{3}{4}$ inches high. Two openings $1\frac{1}{2}$ inches long by $\frac{1}{2}$ inch wide are cut in the side of the cylinder, and covered with phosphor bronze gauze of 60 mesh to the linear inch. The

cylinder rests on the surface of the soil contained in a 2½-inch flower pot, being lightly pressed into the soil for about ¼ of an inch. By this means the structure is held firm and the cage effectively closed at soil level. The top of the lamp globe is closed by means of a cork shive, from which the centre is removed and muslin pasted over the opening. The small flower pot is embedded in soil in a 6-inch flower pot, so as to minimize evaporation and temperature changes. The larger pot is placed in an earthenware saucer, to which water is added as required to keep the soil moist. Sifted surface soil taken from one of the paddocks of the Waite Institute, and mixed with 15% sand, was used. Seedlings of subterranean clover, *Trifolium subterraneum*, were used as the food plant, successive sowings being made in the small pots as required.

When a series of egg batches was required a number of these cages were set up, and a suitable number of adult insects introduced into each globe. Observations were made within 24 hours, and in those cages in which eggs had been laid the insects were removed from the cage, the seedlings taken out, and the small pots containing the eggs *in situ* on the soil were placed in the incubator. Each pot was placed in a saucer (galvanized tin lid), to which water was added as required to keep the soil moist. At the higher temperature (above about 25°C.) frequent attention was necessary, owing to the high evaporation, and doubtless glazed pots having an unglazed bottom would be more satisfactory in this respect.

Another type of cage (type B), resembling type A, but of larger size, was used for observations on egg-lying and incubation of the eggs in the insectary. The zinc cylinder in this case is 3½ inches diameter by 2 inches high, into which an ordinary hurricane lamp globe fits. It is pressed lightly into the soil in a 4-inch flower pot, which is embedded in soil in an 8-inch flower pot placed in a saucer. The top of the globe is closed by a muslin ring of the type described by Imms (2).

Another type of cage (type C) was found particularly useful for observations under the binocular microscope, on hatching of the egg and the behaviour of the early stages of the insect. It consists of a cylinder of phosphor bronze gauze, 60 mesh to the linear inch, 2 inches diameter by 1¾ inches high. It is bound round the top with a strip of zinc ¼ inch wide, and round the bottom by a strip ⅜ inch wide. The cylinder is lightly pressed into the soil and closed at the top with a muslin ring.

EXPERIMENTAL DATA.

Data have been obtained from three series of experiments.

(a) *Series A.*

In this series the eggs were retained in position as laid on the soil in pots. The pots were placed in a multiple temperature incubator which has fourteen

chambers, each 6 inches wide by 4 inches deep and 7 inches high, and divided into two compartments by a horizontal shelf. The incubator was regulated to allow of 14 different temperatures, ranging from 2·5°C. to 35°C. Fluctuations about these temperatures were very small, as is shown in Table 1. Three pots could be placed in each chamber at one time.

Except in a few instances, only one batch of eggs was laid in each pot. In general, the number of eggs in a batch was estimated at about 20 to 30, but in a few cases the batches were larger, and consisted of about 40 to 50 eggs.[1]

The incubation period was reckoned as from the time the first individuals of an egg batch began to hatch out. The work of Peairs (4) on the relation of temperature to development in insects indicated that data from first emergences would give a closer approximation to the development curve than if the mean emergence time for all the individuals from an egg batch were taken.

When at least four individuals from an egg batch had hatched out, which in tests Nos. 19–68 (Table 1) invariably occurred within 12 hours after the emergence of the first individuals, the pot containing the egg batch was removed from the experiment. The number of individuals which hatched in each test was not recorded, but in several instances as many as ten had emerged within a few hours of the emergence of the first individual.

The dense growth of mould fungi over the egg masses in some cases was a serious difficulty, but was partly overcome in later tests by using steam-sterilized soil and watering with distilled water. In those cases where the egg masses were covered with fungal growth they were discarded, as it was felt that the conditions could not be considered similar to those egg batches free from fungal growth.

A further difficulty was experienced in maintaining suitable moisture conditions on the surface soil, particularly at temperatures above about 28°C. This is important in relation to the comparatively few individuals which hatched out in tests Nos. 8–18 (Table 1), and requires further investigation.

Soil moisture is an important factor affecting development of the egg, a factor which has been emphasized by Holdaway (1). This aspect of the problem will be dealt with in a later paper.

The results obtained from 77 tests at various temperatures are given in Table 1.

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[1] The eggs, which are about 0·27 mm. in diameter, are covered by the female with a sticky, brownish fluid excrement which dries on exposure to the air, the eggs being firmly held together, forming a small irregular mound of the same colour as the soil.

TABLE 1.

Incubation Period of Eggs of Sminthurus viridis in Relation to Temperature.

Expt. No.	Date (1930) eggs laid.	Date (1930) eggs hatched.	Incubation period (days).	Temp. °C. during period. Range of Temp.	Mean Temp.
1	July 16	Eggs did not hatch by July 27		34-35	34
2	16				
3	16				
4	July 21	Eggs did not hatch by Aug. 8		33·5-35	34
5	21				
6	22				
7	22				
8	June 26	Eggs did not hatch by July 16		29-31·5	30
9	26				
10	26	3 eggs hatched July 9			
11	July 17	2 eggs hatched July 26			
12	18	No eggs hatched by Aug. 2		29-30	29·7
13	July 23	No eggs hatched by Aug. 8		28-30	28·7
14	23				
15	21				
16	Aug. 6	No eggs hatched by Aug. 25		28-29	28·5
17	6				
18	6				
19	June 27	July 6	9		
20	27	5	8	26-27·5	26·7
21	27	5	8		
22	July 8	17	9		
23	9	17	8	25·5-26	25·6
24	9	17	8		
25	Aug. 10	Aug. 19	8		
26	11	19	8	25-26	25·2
27	12	20	8		
28	June 27	July 6	9	23-24·5	23·8
29	27	5	8	23-24·5	24
30	28	7	9	22-24·5	23·5
31	July 10	19	9	22-23	22·7
32	10	Discarded on July 20, eggs covered with fungus.			
33	20	July 29	9		
34	20	30	10	22-23	22·3
35	20	30	10		
36	Aug. 8	Aug. 18	10		
37	8	18	10	21-22·5	21·8
38	8	19	11		
39	June 28	July 7	9		
40	28	8	10	19·5-21·5	20·6
41	28	8	10		
42	July 11	22	11		
43	11	21	10	19·5-20	20
44	14	25	11		

Expt. No.	Date (1930) eggs laid.	Date (1930) eggs hatched.	Incubation period (days).	Temp. °C. during period. Range of Temp.	Mean Temp.
45	Aug. 7	Aug. 18	11		
46	8	19	11	18·5-20	19
47	11	21	10		
48	June 28	July 9	11		
49	28	10	12	17-19·5	18·3
50	28	10	12		
51	July 14	27	13		
52	14	28	14	17-18	17·4
53	14	30	16		
54	Aug. 6	Discarded on Aug. 25, eggs covered with fungus.			
55	7	Aug. 21	14	16-17·5	16·7
56	7	22	15		
57	June 28	Discarded on July 17, eggs covered with fungus.			
58	29	July 15	16	15-17	15·8
59	29	16	17		
60	July 15	31	16	15-16	15
61	17	Aug. 5	19		
62	July 25	Aug. 16	22	12-13·5	12·7
63	25	17	23		
64	25	Discarded on Aug. 20, eggs covered with fungus.			
65	June 29	Discarded on July 29, eggs covered with fungus.			
66	29	July 23	24	12-14	12·5
67	29	25	26		
68	July 23	Aug. 18	26	10·5-12	11·5
69	June 30	Aug. 17	48		
70	30	Eggs did not hatch by Aug. 23		8-10·5	8·7
71	30				
72	June 30				
73	30	Eggs did not hatch by Aug. 25		5·8	6·3
74	30				
75	June 30				
76	30	Eggs did not hatch by Aug. 25		1·5	2·5
77	30				

(1) *High Temperatures.*

It is seen from Table 1 that the minimum incubation period of 8 days was obtained at a temperature approximating to 25°C.

With tests Nos. 1–7 (34°C.) the eggs had not hatched after periods of 11 and 15 days respectively. On examination of these eggs no embryos were found in them; some of the eggs were black, others appeared normal in colour.

With tests Nos. 8 and 9 (30°C.) the eggs had not hatched after a period of 20 days, although in No. 10 three eggs hatched after 13 days. No further eggs hatched, however. An examination of the eggs showed that in some cases the embryos had partially developed but failed to emerge.

With test No. 11 (29·7°C.) two eggs hatched after 10 days; no further eggs

hatched. With No. 12 (29·7°C.) no eggs had hatched after 14 days. An examination of the eggs in Nos. 11 and 12 showed that in some instances development had taken place. Many eggs showed a distinct equatorial band where the hatching egg normally splits, and contained dead embryos.

With Nos. 13–15 (28·7°C.) the eggs had not hatched after a period of 15 days. An examination of the eggs showed that some development had taken place, but the embryos had been killed. With Nos. 16–18 (28·5°C.) no eggs had hatched in 19 days. Some eggs had undergone partial development, but had been killed.

(2) *Low Temperatures.*

With low temperatures the incubation period was considerably extended, and in Nos. 72–74, with a mean temperature of 6·3°C., no eggs had hatched even after 56 days. This temperature is near the critical cold point, which from figure 1 is seen to be 5·5°C., so that development would be very slow. In Nos. 75–77 the mean temperature of 2·5°C. is well below the critical cold point. In No. 69 (8·7°C.) the eggs commenced to hatch after 48 days, although Nos. 70 and 71 had not hatched by the end of 54 days.

(b) *Series B.*

Ten pots containing steam-sterilized soil were placed in a Hearson seed-germinator incubator maintained at a temperature about 28°C., the variation about this temperature being not greater than 1 degree. Ten egg batches, laid on October 1, were placed, one batch in each pot, in a small depression on the surface of the soil. The latter was watered with distilled water as required.

One egg hatched on October 10 and two more eggs from the same batch on the next day. In another batch one egg hatched on October 12. The individuals died shortly after emerging from the egg. No further eggs had hatched from the whole series of 10 pots by October 20, when the experiment was discontinued.

It should be noted that the conditions in this incubator were very humid, the tray in the bottom of the incubator being kept filled with water.

(c) *Series C.*

A number of observations were made on the incubation period of eggs under the variable conditions of temperature experienced in the insectary in May and June, 1930. Temperature records for the first 10 days of the experiments are not available, but for the remainder of the period, viz., June 9 to June 23, the temperature fluctuated between 44°F. and 82°F., giving an approximate mean temperature of 61°F.

From 23 pots, in which eggs were laid between May 27 and 30, the incubation period varied from 15 to 22 days. An analysis of the results is given below:

With	2	pots	eggs	hatched	after	15	days
"	2	"	"	"	"	16	"
"	2	"	"	"	"	17	"
"	2	"	"	"	"	18	"
"	5	"	"	"	"	19	"
"	5	"	"	"	"	20	"
"	4	"	"	"	"	21	"
"	1	"	"	"	"	22	"

The average incubation period for the series was 18½ days. It is of interest to note from figure 1 that with a temperature of 60°F. an incubation period of about 16 days might be expected. The variability in the incubation periods is doubtless associated with the variable temperature and moisture of the surface soil in the different pots. It may be noted that in the present series of experiments five pots were watered sparingly, the surface soil being practically dry for several days. No eggs had hatched from these pots by the end of 25 days, when the experiment was discontinued.

DISCUSSION OF THE DATA.

The relation of temperature to insect development has been studied by several investigators during recent years. A concise view of the present position regarding the subject is given by Imms (3).

From the evidence available it is evident that the curve of development in relation to temperature in insects is best expressed as a hyperbola, the reciprocal values of which lie along a straight line. The reciprocal curve expresses the velocity of development, and is referred to as the "velocity curve." This curve cuts the base line or temperature axis at a temperature value which is referred to as the "critical cold point" or "threshold of development," which represents the approximate temperature below which development ceases.

These general statements refer to the influence of constant temperatures on development. The hyperbola expresses the distribution of observed points only within certain limits of temperature. As the latter approaches the lethal "high" temperature the points depart from the curve, owing to the retarding effect of the high temperature. Also at low temperatures the results obtained by some experimenters indicate that the observed points depart from the curve before the critical cold point is reached. This lag effect makes the temperature value for the critical cold point somewhat lower than the calculated value where the reciprocal curve cuts the temperature axis.

Recent investigations indicate that, when the temperature values employed are obtained from the mean of variable temperatures, the curve of development does not coincide with the curve obtained when constant temperatures are used. The evidence, however, does not negative the view that the curve of development is best expressed as a hyperbola. It follows, therefore, that when the observed values of the time factor (development period in days) are obtained, and the reciprocal of each value, which represents the percentage development in one

day, is plotted against temperature, the points obtained should approximate to a straight line.

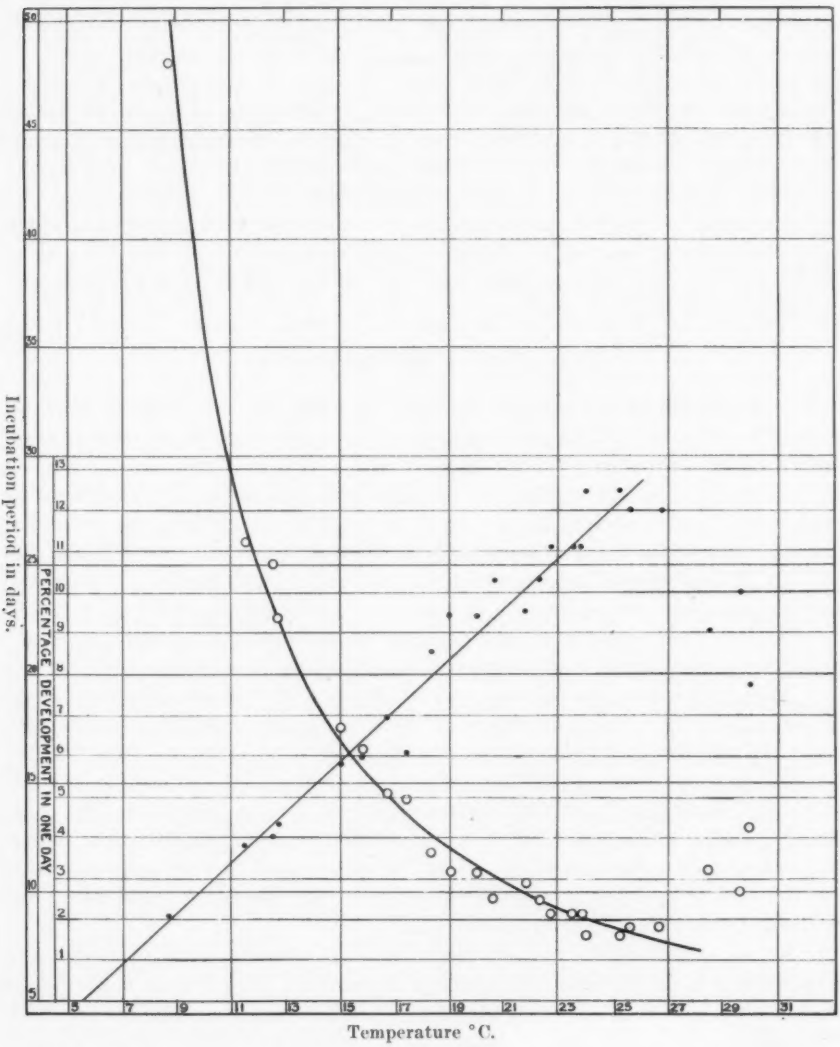

Figure 1. Temperature-development curve and temperature-velocity curve of development of eggs of *Sminthurus viridis* calculated from data shown in Table 2.

O=observed points, incubation period in days plotted against temperature.
●=observed reciprocal values plotted against temperature.

The values for 24 examples taken from the data obtained in my experiments are shown in Table 2. The incubation periods given in column 2 have been obtained from Table 1 by taking the mean developmental period for those tests carried out under similar temperatures.

TABLE 2.

No.	Mean temperature of incubation period, °C.	Incubation period in days.	Reciprocal values. Observed.	Calculated.	Difference.
1	30	13	7·7	—	—
2	29·7	10	10·0	—	—
3	28·5	11	9·1	—	—
4	26·7	8·3	12·0	13·2	—1·2
5	25·6	8·3	12·0	12·5	—0·5
6	25·2	8·0	12·5	12·2	0·3
7	23·8	9·0	11·1	11·4	—0·3
8	24·0	8·0	12·5	11·5	1·0
9	23·5	9·0	11·1	11·2	—0·1
10	22·7	9·0	11·1	10·7	0·4
11	22·3	9·7	10·3	10·4	—0·1
12	21·8	10·3	9·6	10·1	—0·5
13	20·6	9·7	10·3	9·4	0·9
14	20·0	10·7	9·4	9·0	0·4
15	19·0	10·7	9·4	8·4	1·0
16	18·3	11·7	8·6	8·0	0·6
17	17·4	14·3	6·1	7·4	—1·3
18	16·7	14·5	6·9	7·0	—0·1
19	15·8	16·5	6·0	6·4	—0·4
20	15·0	17·5	5·7	5·9	—0·2
21	12·7	22·5	4·4	4·5	—0·1
22	12·5	25·0	4·0	4·3	—0·3
23	11·5	26·0	3·8	3·7	0·1
24	8·7	48·0	2·1	2·0	0·1

The 24 observed reciprocal values given in Table 2 have been plotted against temperature (figure 1). With the exception of the values for Nos. 1, 2, and 3, the distribution of the points obtained, which represents the velocity curve, suggests a straight line. The three exceptional points referred to show the retarding influence of the high temperatures, and have been excluded when calculating the velocity curve. The remaining 21 values have been fitted to a straight line, the calculated formula for which is $Y = 0{\cdot}62x - 3{\cdot}40$, when $x =$ temperature and $y =$ reciprocal value. The zero point of this curve cuts the temperature axis at 5·5°C., which is the "critical cold point."

The differences between the observed points and those on the calculated straight line are shown in the last column of Table 2.

When tested for goodness of fit by the Elderton formula the following value for χ^2 was obtained (5):

$$\chi^2 = S\,\frac{(o-c)^2}{c} = 0{\cdot}492$$

when $o =$ observed point and $c =$ calculated point,

indicating an exceptionally good fit between the observed and calculated results.[2]

[2] I am greatly indebted to Dr. I. Phipps for assistance in the statistical examination of the data.

The hyperbolic curve shown in figure 1 has been calculated from the reciprocal curve. It is represented by the formula $d(t - C) =$ constant, when $d =$ developmental period in days; $t =$ temperature; $C =$ critical cold point $=$ 5·5°C.

SUMMARY.

1. Experiments have been made on the influence of temperature on the incubation period of the eggs of *Sminthurus viridis* L. when ample soil moisture is available.

2. The technique is described.

3. The results of tests on 77 egg batches are given, the incubation period being taken as the period between the laying of the eggs and the mean time of emergence of the first individuals of an egg batch.

4. Examination of the data shows that between 8·7°C. and 26·7°C. the observed reciprocal values approximate closely to a straight line (velocity curve), the calculated formula of which is $y = 0{\cdot}62x - 3{\cdot}40$, when $x =$ temperature and $y =$ calculated point on the line.

5. The zero point of the velocity curve (where it cuts the temperature axis) was found to be 5·5°C. This is the critical cold point.

6. Examination of the observed reciprocal values by Elderton's formula for goodness of fit gives the value $\chi^2 = 0{\cdot}492$, indicating a good fit between the observed and calculated results.

7. Within the range of temperature stated in (4) the curve of development in relation to temperature under the conditions stated, is best expressed as a hyperbola having the formula $d(t - C) =$ constant, where $d =$ developmental period in days; $t =$ temperature; $C =$ critical cold point $=$ 5·5°C.

8. At temperatures above 26·7°C. the developmental period was obtained at 28·7°C., 29·7°C., and 30°C. Only a few individuals hatched out. The mean developmental periods at these temperatures were 13, 10, and 11 days respectively, indicating a retarding influence at these higher temperatures, with which is doubtless associated the variable moisture conditions.

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